The Dissertation Committee for Douglas Wayne Barrett Certifies that this is the approved version of the following dissertation:

Brain Mechanisms of Pavlovian Extinction

Committee:

F. Gonzalez-Lima, Supervisor

Yvon Delville

Michael Domjan

Juan Salinas

Tim Schallert

Brain Mechanisms of Pavlovian Extinction

by

Douglas Wayne Barrett, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2005

Dedication

To my parents, Carol Efferson Cook and Phillip Henry Cook.

To my parents, Carl Wayne Barrett and Glenda Moore Barrett.

I was four times as lucky as most kids, and I couldn't have done it without you!

Acknowledgements

I thank F. Gonzalez-Lima and all current and former members of the FGL Lab for seven years of education, collaboration, and support. I am especially indebted to Jason Berndt and Alison Tannenbaum for teaching me about surgical techniques and tissue sectioning, and to Hemu Nair and Dirk Jones for their tips about behavioral training, autoradiography and imaging. Technical assistance from Amy Kalia, Manfried Robinson, Joel Wommack, and Abby Shindler was also invaluable. Many thanks to Prof. Fritz Henn, who provided our lab with the original breeding stock for the congenitally helpless rats, and also to Jason Shumake, for his wisdom, skill, and merciless editing.

Brain Mechanisms of Pavlovian Extinction

Publication No._____

Douglas Wayne Barrett, Ph.D.

The University of Texas at Austin, 2005

Supervisor: Francisco Gonzalez-Lima

The brain mechanisms that underlie the process of Pavlovian fear extinction are intricate, controversial, and of great interest to those that study learning and memory phenomena. Neural mechanisms of Pavlovian extinction were evaluated with fluoro-deoxy-glucose metabolic mapping of mouse brain regions implicated in fear acquisition and extinction. Subjects were trained to show a conditioned response (CR), freezing, by pairing a tone conditioned stimulus (CS) with a footshock unconditioned stimulus (US) during acquisition, and received CS-alone presentations during extinction. Pavlov's original hypothesis of inhibitory cortical mechanisms underlying extinction was supported by our finding that frontal cortical regions were hyperactive after extinction. Prefrontal activity was correlated with extinction retention behavior and showed evidence of inhibitory coupling with other regions after extinction. Changes in other regions may reflect elements of the original acquisition memory even after extinction, including the CS (auditory system), the US (external cuneate), CS-US contiguity (hippocampus), and expression of the CR (amygdala).

Further behavioral experiments tested individual differences in extinction learning in congenitally helpless rats, a selectively bred strain which shows disruptions in the hypothalamic-pituitary-adrenal (HPA) axis and may model genetic susceptibility to post-traumatic stress disorder. The involvement of corticosterone in extinction learning was tested through the use of metyrapone, a corticosterone synthesis inhibitor. Both congenitally helpless rats and metyrapone-treated mice showed significant deficits in extinction behavior.

We propose that a functional network of mutually interactive brain regions is formed after Pavlovian extinction training, composed of frontal regions, auditory regions, hippocampus, medial thalamus, and brainstem somatosensory systems. This network serves to inhibit the CR while preserving the original CS-US association, and the HPA axis may modulate this network, as evidenced by the extinction deficits seen in both metyrapone-treated and congenitally helpless subjects. This is the first time such a functional network has been demonstrated after Pavlovian extinction.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

The neural mechanisms that underlie the process of Pavlovian fear extinction are intricate, controversial, and of great interest to those that study learning and memory phenomena. This paradigm is a simple model of how a memory is formed, then modified on the basis of experience. Extinction is defined as the reduction of a previously learned response that occurs because a conditioned stimulus (CS) is no longer paired with an unconditioned stimulus (US). In the Pavlovian fear conditioning paradigm, a tone CS is first paired with an aversive footshock US during acquisition, such that presentations of the CS alone will elicit freezing behavior, a defensive conditioned response (CR). During extinction, repeated presentation of the tone alone (in the absence of footshock) will cause the CR to decrease over time.

PAVLOVIAN EXTINCTION: UNLEARNING VS. INHIBITORY MECHANISMS

Extinction behavior was first characterized by Pavlov (1927), who hypothesized that extinction manifests through the formation of inhibitory associations between brain regions that represent the CS and US. These new inhibitory circuits would gradually reduce the occurrence of the CR by counteracting the previously acquired excitatory CS-US association. Pavlov concluded that the original CS-US association remains intact after extinction, and that extinction represents new learning: the formation of new associations instead of destruction of the old. However, others have hypothesized that extinction involves a weakening of the CS-US association (Rescorla & Wagner, 1972) or a reversal of the acquisition process (Richards, Farley, & Alkon, 1984).

However, a substantial body of behavioral evidence indicates that the subject does not return to the naïve state after extinction. Pavlov's (1927) observations of *spontaneous recovery*, in which a previously extinguished CR reappears after the passage of sufficient

time, and *disinhibition*, in which the CR reappears after presentation of a novel stimulus, led to his hypothesis that inhibitory circuits underlie extinction behavior. This hypothesis is also supported by other training paradigms which reveal that the original CS-US association is still present. The *renewal* effect occurs when a subject is returned to the acquisition context, revealing the original CR (Bouton, 2002). *Reinstatement* occurs when a subject is given a US presentation such as a single footshock, and the CR is again made manifest (Falls, 1998). These effects argue for extinction as new learning, not unlearning.

When the CR is viewed as an adaptive response to changing conditions and contingencies, it seems likely that after extinction training, elements of the original association are still present, but are subject to inhibition on the basis of time, context, recent experience, and other factors. In situations where it would be potentially adaptive to express the CR, it would be quicker and more efficient for the brain to simply disinhibit the original learning, rather than relearn the association de novo. This could be reflected in patterns of brain activity which are present after acquisition and persist after extinction, but are not found in subjects who did not undergo acquisition in the first place. However, it could be the case that in some brain regions, extinction training essentially reverses the effect of acquisition, while in other regions, the effects of the original acquisition training remain after extinction training.

NEURAL MECHANISMS OF PAVLOVIAN EXTINCTION

By measuring the metabolic activity of various brain regions implicated in Pavlovian conditioning paradigms, we can test the predictions of each hypothesis. The objective of the first study described here (Barrett, Shumake, Jones, & Gonzalez-Lima, 2003) was to address this question in terms of changes in regional brain metabolism, as measured through fluoro-deoxy-glucose (FDG) autoradiography.

These two dominant hypotheses about what is learned during extinction – inhibition vs. "unlearning" of the CS-US association – lead to different predictions about brain metabolic effects. The "unlearning" position holds that extinction is essentially the opposite of acquisition. This could be reflected by opposite patterns of regional activity after acquisition and extinction. If a particular brain region returns to a metabolic activity level after extinction which is comparable to that in subjects who did not undergo acquisition, this could argue for acquisition and extinction as opponent processes. However, persistent changes common to both acquisition and extinction would be evidence of savings of the CS-US association.

Studies of neural activity provide some support for the hypothesis that extinction is the reversal of acquisition. In a study of the extinction of a phototaxic response in the mollusk *Hermissenda*, Richards et al. (1984) concluded that in terms of electrophysiology and behavior, extinction is the result of the "reversal of the original acquisition processes." While this form of extinction may be the dominant mechanism in simple organisms like invertebrates, more complex brains may employ more complex mechanisms of extinction. Long-term potentiation (LTP) and long-term depression (LTD) have also been proposed as neural models of acquisition and extinction, respectively (Christofi, Nowicky, Bolsover, & Bindman, 1993). The unlearning of the original CS-US association is an implicit assumption of many such studies of neural electrophysiology, which use extinction to demonstrate the reversal of acquisition effects.

There are many similarities between habituation and extinction (Domjan, 2002), in terms of training procedures (one stimulus presented repeatedly) and evoked behavior (a gradual decrease in responding). Both habituation and extinction are susceptible to spontaneous recovery (Bouton, 1991), as well as dishabituation or disinhibition, respectively, in response to a novel stimulus (Domjan, 2002). Neural mechanisms of

extinction may be similar to those of habituation, in which brainstem regions in the reticular activating system showed decreased activity, and cerebellar regions were activated (Gonzalez-Lima, Finkenstadt, & Ewert, 1989b; Gonzalez-Lima, Finkenstadt, & Ewert, 1989a).

The crucial difference between habituation and extinction is that during extinction training, the CS was previously involved in excitatory Pavlovian conditioning during acquisition. While novel stimuli may sensitize the subject enough to respond during dishabituation, novel stimuli do not evoke a CR. In the case of disinhibition, the extinguished CR may be released from neural inhibition by the novel stimulus. It is more likely that regions outside of the brainstem, in CS and US pathways and cortex, are involved in extinction that do not play a role in habituation.

Multiple lines of evidence suggest that extinction is indeed a form of new learning instead of unlearning, even in terms of electrophysiology. A conditioned taste aversion study (McCaughey, Giza, Nolan, & Scott, 1997) found a CS-evoked bursting pattern in the nucleus of the solitary tract which was present after acquisition and persisted after extinction. This nucleus is part of the same sensory system (taste) involved in the training, implying that CS representations may still be reflected in terms of neural activity after extinction training. In our Pavlovian fear conditioning paradigm, the use of a tone CS could lead to long-lasting changes in auditory regions, in both activity and interactions with other regions. However, other effects found outside of the auditory system may reflect aspects of the memory outside of the CS representation, such as the CS-US contiguity, the expression of the CR, and the inhibition of the CR.

The original CS-US contingency from acquisition training may be represented by persistent changes in regions like the hippocampus. This structure is rich in N-methyl-D-aspartate (NMDA) receptors which may play a role in the extinction of conditioned fear

(Szapiro, Vianna, McGaugh, Medina, & Izquierdo, 2003). Use of D-cycloserine as an NMDA agonist can facilitate extinction (Richardson, Ledgerwood, & Cranney, 2004), and NMDA antagonists can retard extinction (Corcoran & Maren, 2001). In addition to contiguity detection, the hippocampus may play a crucial role in the contextual modulation of the memory for fear extinction (Corcoran & Maren, 2004).

Other limbic regions, such as the amygdala, may be specialized for fear acquisition processes in general, and expression of the freezing CR in particular. Many studies have documented the involvement of the central amygdala in CR expression (Helmstetter, 1992; Kim, Rison, & Fanselow, 1993; LeDoux, 1995), in fear-potentiated startle (Davis, Falls, Campeau, & Kim, 1993), and avoidance learning (Poremba & Gabriel, 1997). The central nucleus is the main output zone of the amygdala, and its activity probably reflects the expression of the freezing CR at the time of testing. During the FDG session, the CR has already been extinguished; thus the central amygdala may show opposite effects after acquisition vs. extinction. This may not reflect a general "unlearning" process, however; it is also consistent with the involvement of CR inhibition from outside the amygdala.

This CR inhibition is likely provided by the prefrontal cortex (PFC), which has been extensively implicated as playing a crucial role in inhibitory processes. Lesions of medial PFC disrupt CR extinction in rats (Morgan, Romanski, & LeDoux, 1993), and Milad & Quirk (2002) showed that neurons in infralimbic cortex develop enhanced firing rates during extinction, which are correlated with the decrease in the freezing CR. Stimulation of medial dorsal thalamus, which provides reciprocal projections to PFC, can modify CR extinction as well (Herry, Vouimba, & Garcia, 1999; Herry & Garcia, 2002), indicating that thalamic relays may also be involved in CR inhibition. In humans, damage to frontal regions can result in disinhibited behavior, which can lead to profound changes

in the personality of the individual (Carlson, 2004). If prefrontal cortical regions mediate an inhibitory process, the metabolic activity effects would be present only after extinction, and not after acquisition or pseudorandom training. In addition, an inhibitory influence on other brain regions could manifest as negative-correlation interactions with frontal regions after extinction.

Each of these brain systems may play a role in Pavlovian extinction, in the auditory system (CS representation), the hippocampus (CS-US contiguity), the amygdala (CR expression), and PFC (CR inhibition). Each of these systems may also show different kinds of effects in mean activity, such as excitatory (acquisition) and inhibitory (extinction) effects. Some regions may show opposite ("unlearning") effects after acquisition vs. extinction, and some may show common ("contiguity") metabolic effects that persist after acquisition into extinction. The changing relationships between brain regions may be reflected in inter-regional activity correlations, the result of a functional network of mutually interactive brain regions, formed as a result of CR inhibition.

The objective of the first experiment was to metabolically map the regions of interest in Pavlovian acquisition and extinction, as well as regions implicated in our lab's previous mapping studies of operant extinction (Nair & Gonzalez-Lima, 1999; Nair, Berndt, Barrett, & Gonzalez-Lima, 2001b; Nair, Berndt, Barrett, & Gonzalez-Lima, 2001a), blocking (Jones & Gonzalez-Lima, 2001b), differential inhibition (Jones & Gonzalez-Lima, 2001a), and habituation (Gonzalez-Lima et al., 1989b; Gonzalez-Lima et al., 1989a). By comparing our findings with those from other training paradigms, we hope to elucidate both the neural mechanisms unique to Pavlovian extinction, and how they relate to other inhibitory memory-related changes in brain activity. This experiment is discussed in Chapter 2, and comparisons with previous FDG conditioning studies are discussed in Chapter 5.

BRAIN MAPPING USING FLUORODEOXYGLUCOSE (FDG) AUTORADIOGRAPHY

FDG autoradiography can be used to map changes in brain regional activity because neurons take up glucose for energy metabolism. Both glucose and its analogs, like FDG and 2-deoxy-glucose (2-DG), use the same transport system to cross the blood-brain barrier. In the first step of glucose metabolism, glucose is phosphorylated by hexokinase to glucose-6-phosphate; the same enzyme phosphorylates FDG to FDG-6-phosphate and 2-DG to 2-DG-6-phosphate. But while glucose-6-phosphate is converted to other compounds, DG-6-phosphate analogues are not metabolized further, and instead accumulate inside neurons (Sokoloff, 1992).

The use of FDG has several advantages over other analogs such as 2-DG, as detailed in Gonzalez-Lima (1992). FDG is a better analog of glucose since it is structurally more similar to glucose than 2-DG, and FDG crosses the blood-brain barrier more readily than 2-DG. FDG is trapped inside neurons at a greater rate than 2-DG, since it is more easily phosphorylated. The greater specific activity of FDG makes it a superior tracer as well, since it has six times the radiolabeled carbons as compared to 2-DG. Because FDG administration involves a single intraperitoneal injection, there is no need for more invasive surgical procedures like intravenous injections, catheterization or arterial blood sampling, which could potentially interfere with learning-related changes in brain and behavior. These and other factors have led to the adoption of FDG as the most commonly used glucose analog for learning studies.

Other potential markers of regional activity have disadvantages that make FDG a preferable tracer. In some systems, the associative effects of conditioning may not be discernable by other methods. For example, unlike FDG, immediate-early genes such as c-fos are not “universal” markers; i.e., they do not always reveal activity in some neural systems, like somatosensory systems, which show clear activation using FDG but not c-

fos (Sharp, Sagar, & Swanson, 1993). Thus a marker like c-fos is not adequate to map the US somatosensory effects in our studies.

Another potential problem in the case of mapping studies is that the standard parameters may not have been optimized for driving CS-evoked behavior and the concomitant brain metabolic changes. Our approach to this experimental design problem is two-fold. Behaviorally, we have used larger amounts of stimuli to drive the neural systems; metabolically, we have used a universal metabolic marker, used i.p. administration which extends the effective uptake time to increase signal-to-noise ratio, and used intact animals without bleeding or catheters. When this is done in conjunction with sensitive autoradiographic procedures, the resulting high levels of accumulated evoked activity have allowed our lab to measure learning-related neural changes and correlate these activity readings with behavioral measures of the CR.

Perhaps the biggest advantage of the FDG method is the ability to sample all regions of interest simultaneously in intact brains, allowing the potential expression of both unlearning and inhibitory mechanisms. The FDG method also avoids the problems associated with lesions, electrical stimulation, or invasive surgical procedures, which may change not only the activity of the targeted region, but also nearby fibers of passage and other regions receiving input from the target. These potential confounds may be even more important in studies of Pavlovian extinction, given the extensive functional coupling described in Chapter 2.

CONDITIONED EMOTIONAL RESPONSE

The conditioned response measured in our experiments, suppression of behavior, was originally described by Estes and Skinner (1941), who used a behavioral training paradigm for the study of emotional learning called the *conditioned emotional response* (CER) procedure. The CER most commonly measured in rats and mice is conditioned

suppression (Miller & Spear, 1985). This measure is based on the observation that rats become immobile or "freeze" when they anticipate an aversive stimulus such as electric shock. Fanselow (1989) proposed that this is a natural defensive behavior when a predator is detected. For example, according to Fanselow's predatory imminence theory, when a snake is detected but is not yet about to strike, the defensive behavior of a rat is to "freeze" to decrease the likelihood that the predator will notice the rat. This freezing can be conditioned in anticipation of a predatory strike.

This kind of behavioral/ecological analysis helps to explain why the CR in anticipation to shock (freezing) is significantly different from the unconditioned response (UR) to shock (jumping), and is crucial in the modern view of Pavlov's stimulus-substitution model. The modern view is not that the CS becomes a "substitute" for the US, and thereby elicits the UR. Instead, neural representations of the US and CS-US association are formed after pairing a CS with a US, and the CR is dependent on both these factors (Domjan, 2002). The CS becomes an "anticipatory signal" of the US rather than a "substitute" for the US. Therefore, if a US such as footshock elicits a jump characteristic of predatory strike, the tone CS elicits the freezing behavior displayed in anticipation of a predator's strike.

INDIVIDUAL DIFFERENCES IN EXTINCTION LEARNING

Exposure to inescapable footshock can lead to a deficit in subsequent possible escape responses, an effect called learned helplessness (Overmier & Seligman, 1967). The congenitally helpless strain of rats was selectively bred to exhibit vulnerability to learned helplessness (Henn, Edwards, & Muneyyirci, 1993; Henn & Edwards, 1994). As a result, these subjects are predisposed to exhibit altered behavioral phenotypes evoked by stress (Vollmayr et al., 2004). While the congenitally helpless strain was originally proposed as an animal model of depression (Henn, Johnson, Edwards, & Anderson,

1985), the recent results of the Gonzalez-Lima lab's behavioral characterization of this strain has yielded several similarities with post-traumatic stress disorder (PTSD) as well (Shumake, 2005). An extinction deficit in these rats would further confirm the utility of this strain as an animal model of PTSD.

Our lab has also extensively characterized the baseline brain regional activity of congenitally helpless subjects, using cytochrome oxidase (CO) histochemistry (Shumake, Poremba, Edwards, & Gonzalez-Lima, 2000; Shumake, Edwards, & Gonzalez-Lima, 2001; Shumake, Edwards, & Gonzalez-Lima, 2002; Shumake, Edwards, & Gonzalez-Lima, 2003a; Shumake, Conejo-Jimenez, Gonzalez-Pardo, & Gonzalez-Lima, 2004). Baseline changes were found in regions linked to stress hormone regulation (Shumake et al., 2001) and other regions of interest in Pavlovian extinction. Brain metabolic differences present at birth (Shumake et al., 2004) may render the congenitally helpless strain susceptible to persistent traumatic memories which are resistant to extinction, by disrupting the formation of the functional networks underlying successful extinction learning.

The objective of the second experiment was to test the extinction behavior of the congenitally helpless rat, and interpret these findings on the basis of our CO activity studies. Unlike FDG, the CO method does not measure evoked activity; changes in metabolic capacity occur over a longer time course of days to weeks, and thus the CO method is better suited to measure baseline differences, such as those due to genetic predispositions. The background and results of this experiment are discussed in Chapter 3, and brain metabolic effects which may predispose this strain to show an extinction deficit are discussed in Chapter 5.

ROLE OF CORTICOSTERONE IN PAVLOVIAN EXTINCTION

Disruption of the hypothalamic-pituitary-adrenal (HPA) axis, as seen in the congenitally helpless rat (Shumake et al., 2001), may be involved in extinction deficits. The objective of the third experiment (Barrett & Gonzalez-Lima, 2004) was to test the effect of changing blood corticosterone levels on Pavlovian extinction. Metyrapone is a drug that inhibits glucocorticoid synthesis (corticosterone in rats and mice, cortisol in humans) by inhibiting 11-beta-hydroxylase, a rate-limiting enzyme in corticosteroid synthesis (Haynes Jr., 1990). Metyrapone was successfully used to impair Pavlovian fear acquisition memory (Cordero, Kruyt, Merino, & Sandi, 2002) when administered prior to acquisition. Similar training parameters with metyrapone administered prior to extinction could result in a similar impairment, but reflected in an extinction deficit instead of an acquisition deficit.

Hernandez-Poudevida, McEwen & Quirk (2002) reported that metyrapone prevented the extinction of a conditioned emotional response in rats. However, this finding could not be replicated (G.J. Quirk, personal communication). Generally, the literature suggests that corticosterone administration does not affect learning performance, but dose-dependently enhances memory consolidation (for review, see Lupien & McEwen, 1997). Therefore, in addition to behavioral measurements during extinction performance, two probe trials measured spontaneous recovery (in the extinction context) and the renewal effect (in the acquisition context), to test whether metyrapone differentially affected the performance and retention of Pavlovian extinction. The background and results of this experiment are discussed in Chapter 4.

Chapter 2: Metabolic Mapping of Mouse Brain Activity after Pavlovian Acquisition and Extinction

INTRODUCTION

Pavlov (1927) explained extinction as the formation of inhibitory circuits that reduce the conditioned response (CR) by counteracting the previously acquired excitatory associations between the conditioned stimulus (CS) and the unconditioned stimulus (US). After observing the phenomenon of spontaneous recovery, in which a previously extinguished CR reoccurs after an interval of time, Pavlov concluded that the original CS-US association is not destroyed and that extinction entailed new learning that inhibits the CR. While behavioral phenomena such as spontaneous recovery, rapid reacquisition, renewal and reinstatement (Falls, 1998; Rescorla, 1997) suggest that CS-US associative effects are not completely erased after extinction, other conceptualizations of extinction have described it in terms of the weakening of the CS-US association (Rescorla et al., 1972) or the reversal of the acquisition process (Richards et al., 1984). The present study was in part meant to determine which of these viewpoints is closer to the neural effects of extinction.

As a natural defensive response when a predator is detected, a rodent will stop moving or "freeze" to decrease the likelihood that a predator will notice it (Fanselow, 1989). This freezing behavior can be conditioned as a form of conditioned emotional response (CER) in anticipation of a predatory strike or footshock. Morgan, Romanski and LeDoux (1993) and Quirk, Russo, Barron and Lebron (2000) showed that rats with lesions of the ventromedial prefrontal cortex have across-day retention deficits during extinction of conditioned freezing. However, Gewirtz, Falls and Davis (1997) found that lesions of the rat ventromedial prefrontal cortex failed to affect CER acquisition and

extinction across days. While it has been reported long ago that amygdala lesions disrupt CER acquisition (Goddard, 1964, for review), recent findings suggest that the retention of CER extinction is linked to prefrontal cortex (Milad & Quirk, 2002).

To explore this topic in intact animals, brain changes caused by a tone after extinction of a tone-conditioned CER were assessed with uptake of fluorodeoxyglucose (FDG), a radiolabeled glucose analog. Neural activity can be mapped with FDG because brain cells utilize glucose and its analogs for energy metabolism (Sokoloff, 1992). Different brain metabolic effects of the same tone in groups of control and conditioned mice exposed to the same CS and US served to identify which regional activity changes were caused by the tone after CER extinction. We inferred which neural mechanisms might be unique to Pavlovian extinction by comparing extinction effects with other FDG studies of CR inhibition, such as conditioned inhibition (McIntosh & Gonzalez-Lima, 1993; 1994), instrumental response extinction (Nair et al., 1999; Nair et al., 2001b; Nair et al., 2001a), blocking (Jones et al., 2001b), and differential inhibition (Jones et al., 2001a).

We hypothesized that the largest increase in metabolic activity evoked by the tone after extinction would be in the prefrontal cortex. We also hoped to find changes in other regions, particularly in auditory and limbic networks, due to the savings of CS-US associative effects. The results supported this hypothesis and contradicted the simpler notions of extinction as unlearning or reversal of acquisition.

METHODS AND MATERIALS

Subjects

Subjects were 48 male CBA/J mice, 5 weeks of age when delivered from the supplier (Jackson Laboratory, Bar Harbor, ME). An initial pilot study with 16 mice was conducted to determine the parameters for a training paradigm that would result in the extinction of freezing behavior.

For the subsequent FDG study, 32 naïve subjects were divided into three groups, with n = 11 in the extinction group, n = 11 in the non-extinction group, and n = 10 in the pseudorandom group. Subjects were housed in AALAC-approved facilities under standard laboratory conditions, two to a cage, with a 12:12 light:dark cycle and free access to food and water. Subjects were handled every day for seven days prior to the start of training. All animal experimentation was approved by the University of Texas Institutional Animal Care and Use Committee and complied with all applicable Federal and NIH guidelines.

Apparatus

Phase I (the acquisition phase) of the experiment occurred in context A. The training apparatus for the acquisition phase consisted of a conditioning chamber (22 cm x 14 cm x 22 cm, MED Associates Inc., St. Albans, VT), enclosed in a sound-attenuated box, illuminated by a red light. Two sides of the chambers were aluminum, with clear plexiglass for the front, back and top. Tones were generated by two Wavetek Sweep/Modulation generators (Wavetek, San Diego, CA), and presented through speakers mounted in the top of each chamber. The acoustic CS was a frequency-modulated tone of 1-2 kHz, 2 sweeps per second, 15 seconds in duration, with an

intensity of 65 dB, measured at the center of the chamber's floor. The US was a footshock of 0.5 mA, 0.75 seconds in duration, delivered through metal bars separated by 0.6 cm forming the floor of the chamber, which was wired to a Lafayette Instruments Master Shocker (Lafayette Instrument Co., Lafayette, IN). Presentations of stimuli were controlled by computer programs, created using the MED-PC for Windows programming language (MED Associates, Inc). Between sessions the operant chambers were washed with soap.

Phase II (the extinction training) and the FDG uptake period of the experiment utilized a different context (context B): a clear plastic cage (19 cm x 25 cm x 15 cm), with a speaker mounted in the lid, placed in an illuminated testing room. Between sessions, each extinction box was washed and swabbed with iodine, to provide a distinctive olfactory environment.

Behavioral training

Conditioned behavior

The CER measured was freezing behavior, operationally defined as the mouse having all four feet on the floor, with minimal head movements and shallow, rapid breathing, for at least three seconds. The CS was conditioned to elicit a freezing response through pairing with the US. Each 15-second tone CS was divided into five 3-second bins, with the subject's behavior scored for each of the five bins. Behavior was recorded for the 15 seconds prior to the onset of the tone, as well as the subsequent 15 seconds during presentation of the tone CS, to provide a comparison between activity with and without the CS. An experimenter unaware of the subject's group did the behavioral recordings.

Experimental design

Prior to training, subjects were randomly assigned to one of 3 groups: extinction, non-extinction, and pseudorandom (Table 2.1). The first two groups underwent tone-shock pairing, but one group was trained to extinguish the CER while the other was not. The pseudorandom group underwent no repeated tone-shock pairings and developed no CER. This experimental design permitted dissociating among (1) the brain effects of CER extinction (extinction group vs. non-extinction and pseudorandom), (2) the effects of CER expression (non-extinction group vs. extinction and pseudorandom), and (3) the effects of tone-shock pairing (extinction and non-extinction groups vs. pseudorandom).

Phase I

Days 1-2 of training consisted of habituation to context A for all subjects. During this period, each subject explored the chamber for one hour, with no tones or shocks. Days 3-4 of training were again conducted in context A, and consisted of acquisition training for two groups (extinction and non-extinction groups) and pseudorandom tone and shock presentations for the pseudorandom group. Daily acquisition training consisted of four tone-shock presentations over 15 minutes, with intertrial intervals ranging from 2, 2.5, 3, 3.5, and 4 minutes, randomly shuffled by the MED-PC program. During each trial of acquisition training, the 15-second tone and 0.75-second footshock co-terminated. Daily pseudorandom training consisted of alternating presentations of 4 tones and 4 shocks over 15 minutes, with intervals ranging from 1, 1.5 and 2 minutes, randomly shuffled by the MED-PC program. The pseudorandom training also included exactly one paired presentation of CS-US over the two days, to prevent the tone from being conditioned as a safety signal.

Table 2.1: Experimental design

Groups	Phase I Days 3-4	Probe CER Day 5	Phase II Days 5-6	Probe CER Day 7	FDG Test Day 7
1. Extinction (Extinction after acquisition)	T → S	CER	T	No CER	T
2. Non-extinction (No extinction after acquisition)	T → S	CER	No T	CER	T
3. Pseudorandom (No extinction, no acquisition)	T, S	No CER	T / No T	No CER	T

T, 1 to 2 kHz FM tone sweep (15 sec); *S*, 0.5 mA footshock (0.75 sec); →, temporally contiguous tone/shock; *comma*, pseudorandomly timed discontiguous tone and shock. *CER*, freezing behavior scored during 15-sec tone.

Phase II

Since our goal was to examine the neural effects evoked by the tone rather than by context A, all subsequent steps (probe trials, extinction training, and FDG uptake) were conducted in context B to minimize the effects of excitatory conditioning to context A. At the start of Day 5, each subject was placed in context B for 15 minutes and given probe trials, consisting of 4 presentations of the CS. Behavior was scored as described above. Days 5-6 of training involved 1-hour sessions in context B, and consisted of tone alone presentations in the extinction group and no tones in the non-extinction (acquisition alone) group. For the pseudorandom group, mice received either tone alone or no tone. No behavioral or brain differences were found in the pseudorandom subgroups so these mice were all treated as one group for the statistical analysis. Tone alone consisted of 60 presentations of the 15-second tone CS in one hour, with 45 seconds between each CS presentation. No tone consisted of one hour in context B with no presentations of CS or US.

FDG test

Day 7 consisted of probe trials, FDG administration, and exposure to the CS. Probe trials were conducted as described above and compared to the Day 5 probe trial results, to verify that the CER was still present in the non-extinction group and extinguished in the extinction group. This was followed by injection of FDG: subjects received an IP injection of 18 μCi/100 grams body weight of [^{14}C(U)]2-fluoro-2-deoxy-D-glucose (FDG); (specific activity, 300 mCi/mmol, American Radiolabeled Chemicals) in 0.1 ml of sterile saline. Subjects weighed a mean of 25 grams at the time of FDG administration. Subjects were immediately placed in context B (the extinction context)

and exposed to the tone in a 5-second-on, 1-second-off cycle for 45 minutes, a period chosen from our pilot study to preserve the CER and optimize FDG uptake to the tone. Since most of the FDG uptake is trapped in the first 10 minutes after injection, the majority of the FDG label reflects the subject's initial response to the CS, consisting of freezing in the non-extinction group and no behavioral differences in the extinction and pseudorandom groups. Then subjects were removed from the room and quickly decapitated with a guillotine. Each brain was rapidly removed and frozen in -40°C isopentane for approximately 3 minutes.

FDG autoradiography

The standard FDG autoradiographic procedure (Gonzalez-Lima, 1992, for review) was chosen for metabolic mapping because it has several advantages over 2-deoxy-glucose (2-DG) and because FDG has been used in all of our previous conditioning studies. Briefly, FDG is structurally more similar to glucose than 2-DG, and is thus a better glucose analog; the blood-brain barrier is significantly more permeable to FDG than 2-DG; FDG phosphorylation in the brain is significantly greater than that of 2-DG, and thus FDG is trapped more readily by the brain than 2-DG. Since all six carbons of FDG are radiolabeled, it also has a greater specific activity as a tracer than 2-DG with one radiolabeled carbon.

Sections of the brain were cut at 40 microns at -20°C on a Reichert-Jung 2800 Frigocut E cryostat. Sections used for FDG autoradiography were picked up on slides and immediately dried on a hot plate at 60°C. Slides were affixed to posterboard with double-sided tape, along with plastic standards of known ^{14}C concentration (Amersham Pharmacia Biotech, Arlington Heights, IL) that were used to calibrate the imaging system and report ^{14}C concentrations. In a darkroom, the slides were closely apposed to Kodak

EB-1 film and tightly packed inside Kodak X-O-Matic cassettes (Eastman Kodak, Rochester, NY) for 2 weeks. Films were developed in Kodak D-19 for 2 min, rinsed in stop bath for 1 min, and fixer for 8 min. After development, films were hung to dry, labeled, and stored in protective covers.

Image analysis

FDG uptake was quantified using JAVA image analysis software (version 1.4, Jandel Scientific, San Rafael, CA). The film was placed on a light box and artifact-free images were captured through a black-and-white video camera (Javelin model JE2362, Meyers Instruments, Houston, TX). Images were digitized and corrected for film background and optical distortions from the camera through subtraction of the background. The absolute gray levels of the ^{14}C standards on each film were used to create a calibration curve unique to each individual film, which allowed all optical density measurements taken from brain regions to be automatically expressed in terms of isotope incorporation per gram of brain tissue (nCi/g). The mouse brain atlases by Paxinos and Franklin (2001) and Slotnick and Leonard (1975) were used to locate each region measured. FDG incorporation was measured from 17 auditory and 64 extra-auditory brain regions (Fig. 3).

Three adjacent sections at 80-micron intervals were measured for each region of interest (ROI), with four readings taken in each section. The size of the measurement window was adjusted to allow four non-overlapping measurements covering the entire region. In addition to measuring activity in the regions of interest, readings from white matter (the optic tract) were taken to serve as covariates.

Statistical analyses

Behavior

Changes in behavior across training days and differences in behavior across groups were evaluated based on probe trial data from Days 5 and 7. Evidence of extinction was evaluated by comparing the behavior of the extinction group between Days 5 and 7 (between which the subjects undergo extinction training) using an analysis of variance (ANOVA) with tests for simple effects where appropriate; differences between groups were analyzed with ANOVA as well.

Mean brain activity

Group means of FDG uptake were analyzed as in our recent study of blocking of tone conditioning (Jones et al., 2001b). To reduce variability resulting from individual differences in FDG uptake unrelated to the experimental paradigm, white matter readings were used as covariates in an analysis of covariance (ANCOVA) of brain activity measurements. As there was no statistically significant difference in the white matter readings between groups, the ANCOVA can use the covariate to compensate for small variations in isotope incorporation across individuals. The significance level was set at 99% confidence ($p < 0.01$). Mean activity readings were then expressed in nanocuries per gram of tissue for each ROI, adjusted by the covariate white matter readings, with 99% confidence intervals for each group. If the measurements for two groups are both outside the other's 99% confidence interval, the effect is considered significant. Each ROI is treated as independent of the others, and one simply accepts that 1 of 100 comparisons (for $p = 0.01$) may be Type 1 errors. This is a standard procedure in neuroimaging studies that cannot apply any correction for multiple comparisons due to the large number of ROIs being sampled (Nobrega, 1992). Optical density measurements

of the cochlear nuclei in one pseudorandom group subject were not available, and a linear interpolation was used to create two data points for the ventral and dorsal cochlear nuclei in this control subject. This did not change any pattern of significance in the pseudorandom group correlations among the cochlear nuclei, but served to provide a uniform comparison for the other two groups.

Inter-regional correlations

Since extinction of instrumental behavior is manifested as neural changes in interactivity, the functional relationships among the regional brain activity data were analyzed in terms of pairwise correlations within each group, as in our FDG study of extinction of instrumental behavior (Nair et al., 1999). For the inter-regional correlation analysis, Pearson product-moment correlations were computed, including pairwise comparisons of each region that showed a mean difference between groups as revealed by the ANCOVA means analysis. Optical density measurements of the optic tract were again used as a covariate to control for individual differences in film processing. To ensure the reliability of correlations, a jackknife procedure was performed in which each individual subject was dropped from a group, and then correlations were calculated again without that subject's data. This procedure is iterated until each subject has been sequentially dropped and the analysis performed again. Correlations were considered to be "reliably" significant only if they remained significantly ($p < 0.01$) different from zero throughout all iterations. This is a conservative method sensitive to outliers that avoids inflated Type 1 errors due to the large number of inter-regional correlations computed relative to the sample sizes.

Brain-behavior correlations

Correlations between brain activity and behavioral measurements of freezing during probe trials were also calculated for regions showing major extinction effects, using an *extinction retention index* defined as the freezing behavior ratio between post-acquisition and post-extinction probe trials (Phase I freezing / Phase II freezing). A high index reflects a greater reduction of freezing behavior, and therefore more behavioral extinction. This value was calculated for both the extinction and non-extinction groups and correlated with the brain activity of these two groups. (The pseudorandom group was not included because they did not develop a CER.) Positive brain-behavior correlations reflect a linear relationship between increased regional brain activity and reduction of the CER.

RESULTS

Behavioral results

The CER demonstrated by the CBA/J mice was quite resilient; two one-hour extinction sessions were required to extinguish the freezing behavior. Figure 2.1 shows freezing scores during tone and no tone presentations for the first and second extinction sessions, which demonstrates that a specific CER to the tone is present during the 45-minute period selected for the FDG session.

Average freezing counts during tone-alone probe trials after both Phase I acquisition and Phase II extinction training are summarized in Figure 2.2. Analysis of the probe trial behavioral data with ANOVA confirmed the significant increases in freezing behavior in the extinction ($F(1,19) = 41.94$; $p < 0.01$) and non-extinction ($F(1,19) = 40.41$; $p < 0.01$) groups after Phase I associative tone-shock training, compared to

Figure 2.1: Freezing behavior during first day (A) and second day (B) of extinction sessions. The CER was scored during tone alone presentations and averaged across 8-minute bins. White bars represent counts of freezing during tone CS; Black bars represent counts of freezing with no tone CS, as measured prior to tone onset.

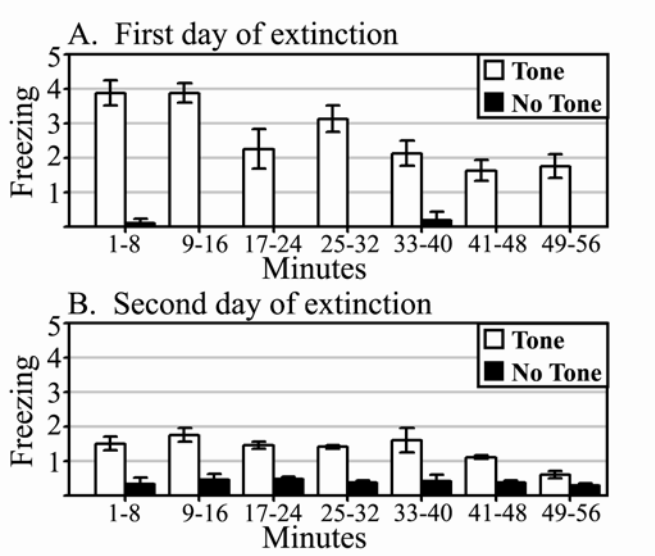
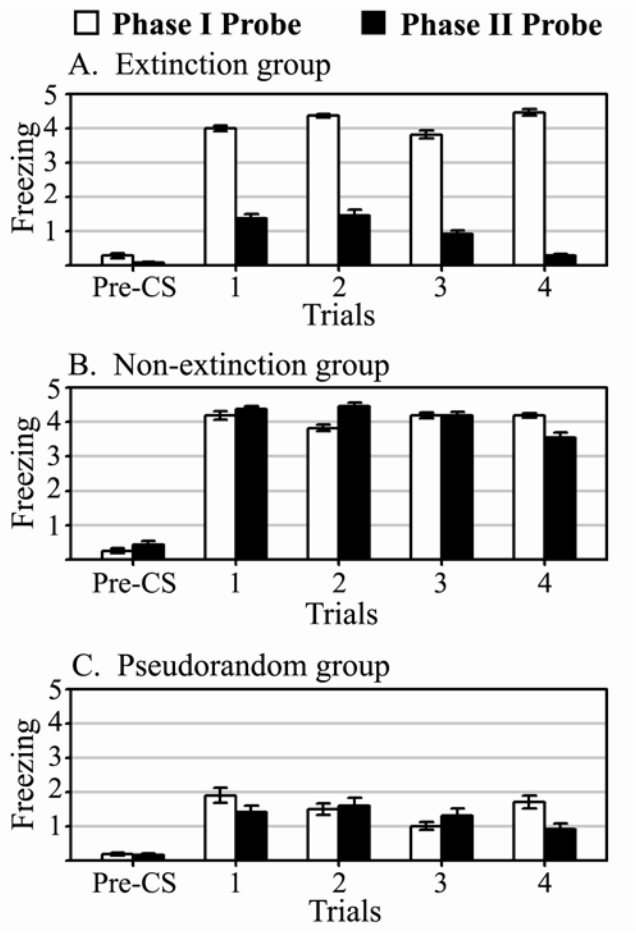


Figure 2.2: Probe trial freezing behavior. Data for each of four tone CS presentations during probe sessions I and II are shown. White bars represent counts of freezing during Probe I (post-acquisition); Black bars represent counts of freezing during Probe II (post-extinction). Pre-CS freezing was measured during the 15 seconds prior to tone onset, and averaged across trials.



pseudorandom. After Phase II training, the extinction group showed a significant ($F(1,20) = 123.02$; $p < 0.01$) decrease in freezing behavior relative to the non-extinction group. The non-extinction group continued to respond with significantly more freezing behavior than the pseudorandom ($F(1,19) = 27.95$; $p < 0.01$). There was no significant difference in freezing behavior between the extinction and pseudorandom groups after extinction training. Finally, there was no freezing behavior to the context during the tone-off periods preceding each trial (pre-CS), demonstrating that contextual excitatory effects were not transferred from context A to B, and that the CER observed was evoked by the tone.

Mean brain activity results

Out of 81 total regions of interest measured, 34 showed significant effects ($p < 0.01$) from the ANCOVA. In all, 14 out of 17 auditory regions showed a significant activity increase due to acquisition training, and 20 out of 64 extra-auditory regions showed significant effects due to acquisition, extinction or both. In general, three classes of effects were observed: (1) Elevated activity in the extinction group; (2) Elevated activity in the non-extinction group; and (3) Extinction and non-extinction groups greater than pseudorandom group. A summary of these effects is listed in Table 2.2 and the regions are illustrated in Figure 2.3. Means, standard errors, and 99% confidence intervals for all 81 regions sampled are listed in Appendix Tables 6.1 and 6.2, for extra-auditory and auditory regions, respectively.

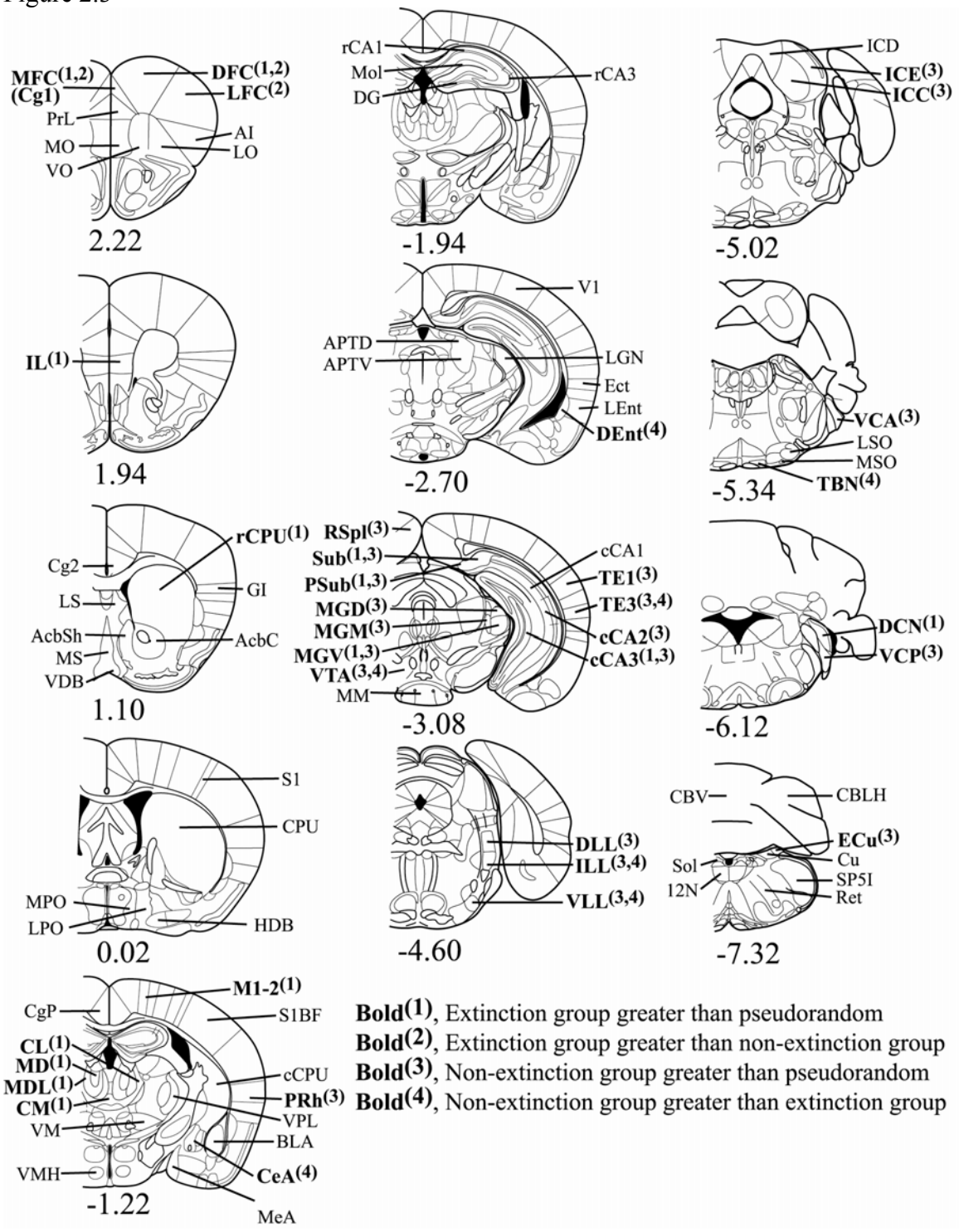
Table 2.2: Significant group effects in mean activity values.

	Extinction	Non-extinction	Pseudorandom
I. Elevated activity in the extinction group			
1) Extinction group greater than both non-extinction and pseudorandom			
Medial frontal cortex (Cg1)	481 ± 18	409 ± 17	408 ± 16
Dorsal frontal cortex (DFC)	478 ± 21	414 ± 19	411 ± 18
2) Extinction group greater than pseudorandom			
Infralimbic cortex (IL)	364 ± 19	323 ± 17	295 ± 16
Medial dorsal thalamus (MD)	459 ± 14	443 ± 14	409 ± 15
Medial dorsal lateral thalamus (MDL)	508 ± 13	481 ± 13	450 ± 14
Centrolateral thalamus (CL)	519 ± 13	493 ± 13	472 ± 13
Centromedial thalamus (CM)	457 ± 12	436 ± 12	415 ± 13
Rostral caudate-putamen (rCPU)	520 ± 28	463 ± 25	435 ± 24
Medial parietal cortex (M1-2)	461 ± 11	445 ± 11	426 ± 12
Dorsal cochlear nucleus (DCN)	563 ± 19	561 ± 19	508 ± 26
3) Extinction group greater than non-extinction			
Lateral frontal cortex (LFC)	443 ± 21	380 ± 19	383 ± 18
II. Elevated activity in the non-extinction group			
1) Non-extinction group greater than both extinction and pseudorandom			
Auditory cortex, ventral (TE3)	366 ± 19	411 ± 20	355 ± 19
Lateral lemniscal nucleus, intermediate (ILL)	411 ± 24	489 ± 32	377 ± 29
Lateral lemniscal nucleus, ventral (VLL)	415 ± 21	476 ± 28	380 ± 25
Ventral tegmental area (VTA)	269 ± 12	327 ± 11	287 ± 10
2) Non-extinction group greater than pseudorandom			
Auditory cortex, dorsal (TE1)	419 ± 19	429 ± 20	385 ± 19
Medial geniculate nucleus, dorsal (MGD)	359 ± 14	383 ± 14	333 ± 16
Medial geniculate nucleus, medial (MGM)	389 ± 13	410 ± 13	362 ± 15
Inferior colliculus, external (ICE)	473 ± 23	509 ± 23	428 ± 25
Inferior colliculus, central (ICC)	749 ± 30	765 ± 30	689 ± 33
Lateral lemniscal nucleus, dorsal (DLL)	357 ± 20	373 ± 27	314 ± 25
Ventral cochlear nucleus, anterior (VCA)	489 ± 17	515 ± 17	449 ± 22
Ventral cochlear nucleus, posterior (VCP)	498 ± 22	520 ± 22	436 ± 29
Perirhinal cortex (PRh)	315 ± 9	333 ± 9	292 ± 9
Retrosplenial cortex (RSpl)	384 ± 16	406 ± 17	355 ± 18
Posterior hippocampus, CA2 (cCA2)	322 ± 12	342 ± 13	299 ± 13
External cuneate nucleus (ECu)	578 ± 22	605 ± 22	538 ± 22
3) Non-extinction group greater than extinction			
Trapezoid body nucleus (TBN)	339 ± 15	373 ± 15	342 ± 21
Central amygdala (CeA)	175 ± 15	226 ± 13	202 ± 13
Deep entorhinal cortex (DEnt)	226 ± 12	279 ± 11	246 ± 10
III. Extinction and non-extinction groups greater than pseudorandom			
Posterior hippocampus, CA3 (cCA3)	292 ± 11	309 ± 12	263 ± 12
Subiculum (Sub)	409 ± 14	414 ± 15	370 ± 15
Presubiculum (PSub)	400 ± 13	414 ± 14	365 ± 15
Medial geniculate nucleus, ventral (MGV)	423 ± 14	427 ± 14	383 ± 16

Group differences are significant at $p < .01$ after ANCOVA. Regional FDG uptake means ± SEs are expressed as nanocuries of FDG incorporation per gram tissue wet weight.

Figure 2.3: Coronal brain diagrams of locations of regions of interest by Bregma level. The significant mean activity differences ($p<0.01$) observed in each region are indicated in boldface. Anterior-posterior Bregma coordinates are indicated below each diagram. (Section diagrams were reproduced with permission from The Mouse Brain in Stereotaxic Coordinates, G. Paxinos and K. Franklin, New York: Academic, 2001, CD-ROM). MFC, medial frontal cortex (Cg1 in Paxinos and Franklin, 2001); PrL, prelimbic frontal cortex; MO, medial orbital cortex; VO, ventral orbital cortex; DFC, dorsal frontal cortex; LFC, lateral frontal cortex; AI, agranular insular cortex; LO, lateral orbital cortex; IL, infralimbic cortex; Cg2, anterior cingulate; LS, lateral septal nucleus; MS, medial septal nucleus; AcbSh, accumbens shell; AcbC, accumbens core; VDB, ventral diagonal band nucleus; rCPU, caudate-putamen rostral; GI, granular insular cortex; MPO, medial preoptic area; LPO, lateral preoptic area; HDB, horizontal limb of diagonal band posterior; S1, parietal cortex anterior; CPU, caudate-putamen middle; CgP, posterior cingulate; CL, central lateral thalamic nucleus; MD, medial dorsal thalamic nucleus; MDL, medial dorsal lateral thalamic nucleus; CM, centromedial thalamic nucleus; VM ventromedial thalamic nucleus; VMH, ventromedial hypothalamus; M1-2, parietal cortex medial; S1BF, parietal cortex lateral; cCPU, caudate-putamen caudal; PRh, perirhinal cortex anterior; VPL, ventral posterior lateral thalamic nucleus; BLA, basolateral amygdala; CeA, central amygdala; MeA, medial amygdala; rCA1, anterior hippocampus CA1; rCA3, anterior hippocampus CA3; DG, dentate gyrus; Mol, hippocampal molecular layers; APTD, anterior pretectal area dorsal; APTV, anterior pretectal area ventral; V1, visual cortex; LGN, lateral geniculate nucleus; Ect, ectorhinal cortex posterior; LEnt, lateral entorhinal cortex; DEnt, deep entorhinal cortex; RSpl, retrosplenial cortex; Sub, subiculum; Psub, presubiculum; MGD, medial geniculate nucleus dorsal; MGM, medial geniculate nucleus medial; MGV, medial geniculate nucleus ventral; VTA, ventral tegmental area; MM, mammillary bodies; cCA1, posterior hippocampus CA1; cCA2, posterior hippocampus CA2; cCA3, posterior hippocampus CA3; TE1, auditory cortex dorsal; TE3, auditory cortex ventral; DLL, lateral lemniscus nucleus dorsal; ILL, lateral lemniscus nucleus intermediate; VLL, lateral lemniscus nucleus ventral; ICD, inferior colliculus nucleus dorsal; ICE, inferior colliculus nucleus external; ICC, inferior colliculus nucleus, central; VCA, ventral cochlear nucleus anterior; LSO, lateral superior olivary nucleus; MSO, medial superior olivary nucleus; TBN, trapezoid body nucleus; DCN, dorsal cochlear nucleus; VCP, ventral cochlear nucleus posterior; CBV, cerebellum vermis; CBLH, cerebellum lateral hemisphere; ECu, external cuneate nucleus; Cu, cuneate nucleus; SP5I, spinal trigeminal nucleus; Ret, medullary reticular formation; Sol, solitary tract nucleus; 12N, hypoglossal nucleus.

Figure 2.3



Bold(1), Extinction group greater than pseudorandom
Bold(2), Extinction group greater than non-extinction group
Bold(3), Non-extinction group greater than pseudorandom
Bold(4), Non-extinction group greater than extinction group

Elevated activity in the extinction group

1) Extinction group greater than both non-extinction and pseudorandom groups.

(Figure 2.4) The most prominent effect revealed by the mean activity analysis was the significantly elevated metabolism of prefrontal regions in the extinction group. There was a trend for elevated FDG uptake throughout prefrontal cortex, with medial prefrontal and dorsal frontal regions showing significant increases (15-18%) relative to both the pseudorandom and non-extinction groups.

2) Extinction group greater than pseudorandom group.

(Figure 2.5) Other regions showed greater activity in the extinction group as compared to the pseudorandom but not the non-extinction group. The infralimbic cortex showed the largest increase with 23% greater FDG uptake than the corresponding value in the pseudorandom group. Neural activity in the medial thalamus increased in a widespread fashion (10-13%), particularly for the medial dorsal thalamic nuclei, which are reciprocally connected with the frontal regions. Also affected were the centromedial and central lateral nuclei, which make widespread, diffuse modulatory connections throughout the cortex. Increased metabolism in the extinction group was also seen in the rostral caudate-putamen (19%) and medial parietal cortex (8%), which serve as a part of the mouse's sensorimotor US representation, and in the dorsal cochlear nucleus (11%) which is part of the auditory CS representation.

3) Extinction group greater than non-extinction group.

(Figure 2.4) While the lateral prefrontal cortex only approached statistical significance relative to pseudorandom, it was significantly higher (17%) than the non-extinction group, which was similar in activity to pseudorandom. However, other prefrontal regions such as prelimbic and orbital regions showed no significant effects.

Figure 2.4: Frontal regions of mouse brain showing increased FDG uptake in the extinction group (ANCOVA, $p < 0.01$). Means +/- SEs are expressed as nanocuries of isotope incorporation per gram brain tissue, with white matter readings from optic tract as covariates. Abbreviations: MFC, medial frontal cortex; DFC, dorsal frontal cortex; LFC, lateral frontal cortex.

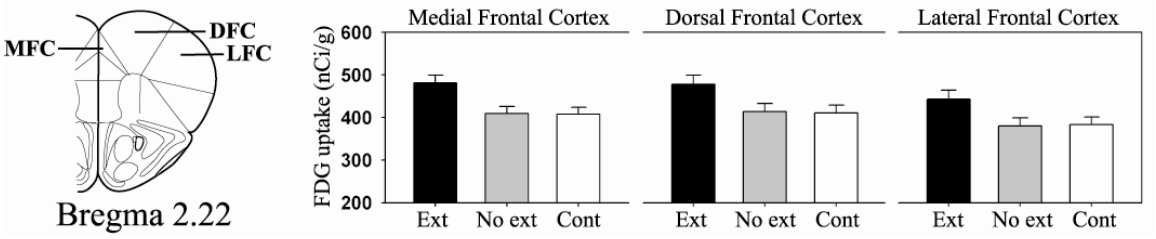
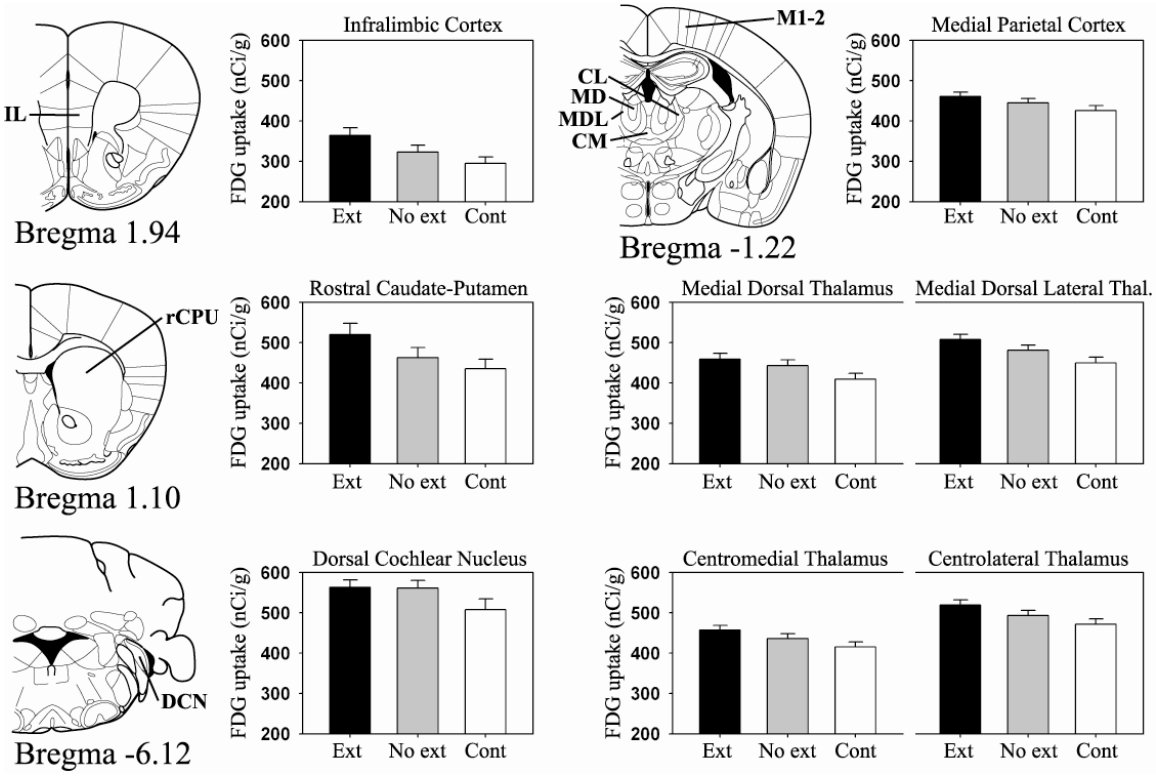


Figure 2.5: Regions of mouse brain showing increased FDG uptake in the extinction group, relative to pseudorandom group (ANCOVA, $p < 0.01$). Means and SEs are expressed as nanocuries of isotope incorporation per gram brain tissue, with white matter readings from optic tract as covariates. Abbreviations: IL, infralimbic cortex; rCPU, rostral caudate-putamen; DCN, dorsal cochlear nucleus; M1-2, medial parietal cortex; MD, medial dorsal thalamic nucleus; MDL, medial dorsal lateral thalamic nucleus; CM, centromedial thalamic nucleus; CL, centrolateral thalamic nucleus.



Elevated activity in the non-extinction group

1) Non-extinction group greater than both extinction and pseudorandom groups.

(Figure 2.6) Parts of the auditory system, including auditory cortex (TE3) and both the intermediate and ventral nuclei of the lateral lemniscus, showed elevated activity in the non-extinction group, relative to both extinction (12-19%) and pseudorandom (16-30%) groups. Another region showing this excitatory effect (14-22%) is the ventral tegmental area (VTA), which may be involved in the expression of the CER. These non-extinction group increases may reflect tone-evoked CER excitatory components greater than any CS-US associative savings common to extinction and non-extinction groups.

2) Non-extinction group greater than pseudorandom group.

(Figure 2.7) The most consistent examples of this effect were seen in the auditory system, at virtually every level, from the primary auditory cortex (TE1, 11%) through medial geniculate nuclei (13-15%), the inferior colliculus (11-19%), dorsal lateral lemniscus nucleus (19%), to ventral cochlear nuclei (15-19%). For the non-extinction group, the tone CS acquired an excitatory salience not evident in the pseudorandom group, and as such, the auditory system showed increased neuronal metabolism at all levels of processing.

Similar excitatory effects (14%) were found in perirhinal and retrosplenial cortices, and the CA2 region of the hippocampus. The external cuneate nucleus of the brainstem, which relays somatosensory information, also showed a 13% increase in metabolism in the non-extinction group.

Figure 2.6: Regions of mouse brain showing increased FDG uptake in the non-extinction group, relative to both extinction and pseudorandom group (ANCOVA, $p < 0.01$). Means +/- SEs are expressed as nanocuries of isotope incorporation per gram brain tissue, with white matter readings from optic tract as covariates. Abbreviations: TE3, ventral auditory cortex; VTA, ventral tegmental area; ILL, intermediate lateral lemniscal nucleus; VLL, ventral lateral lemniscal nucleus.

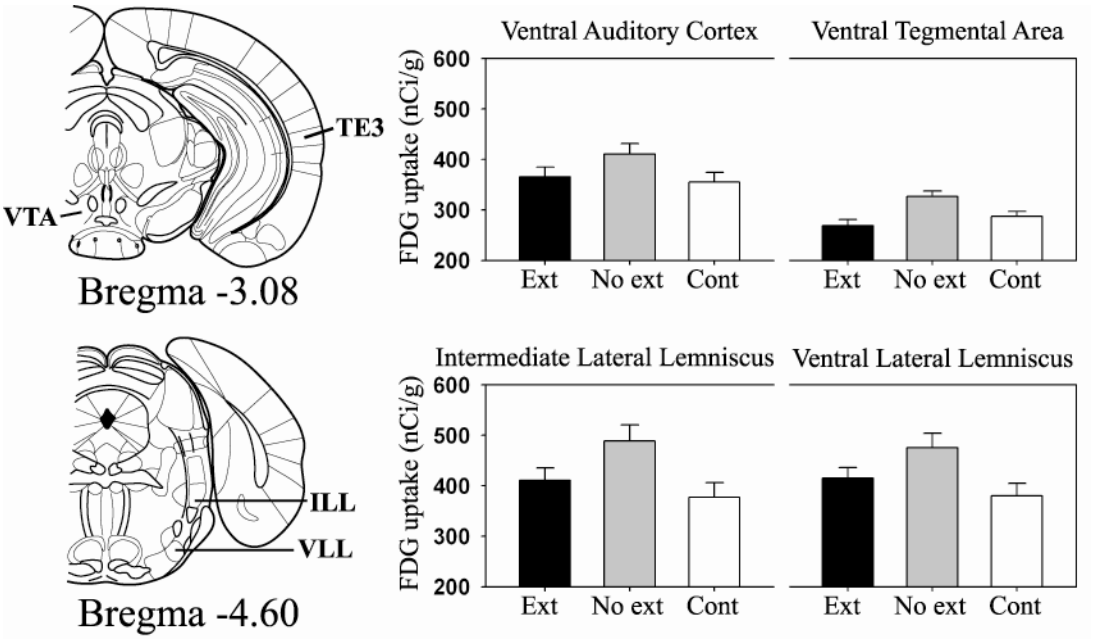
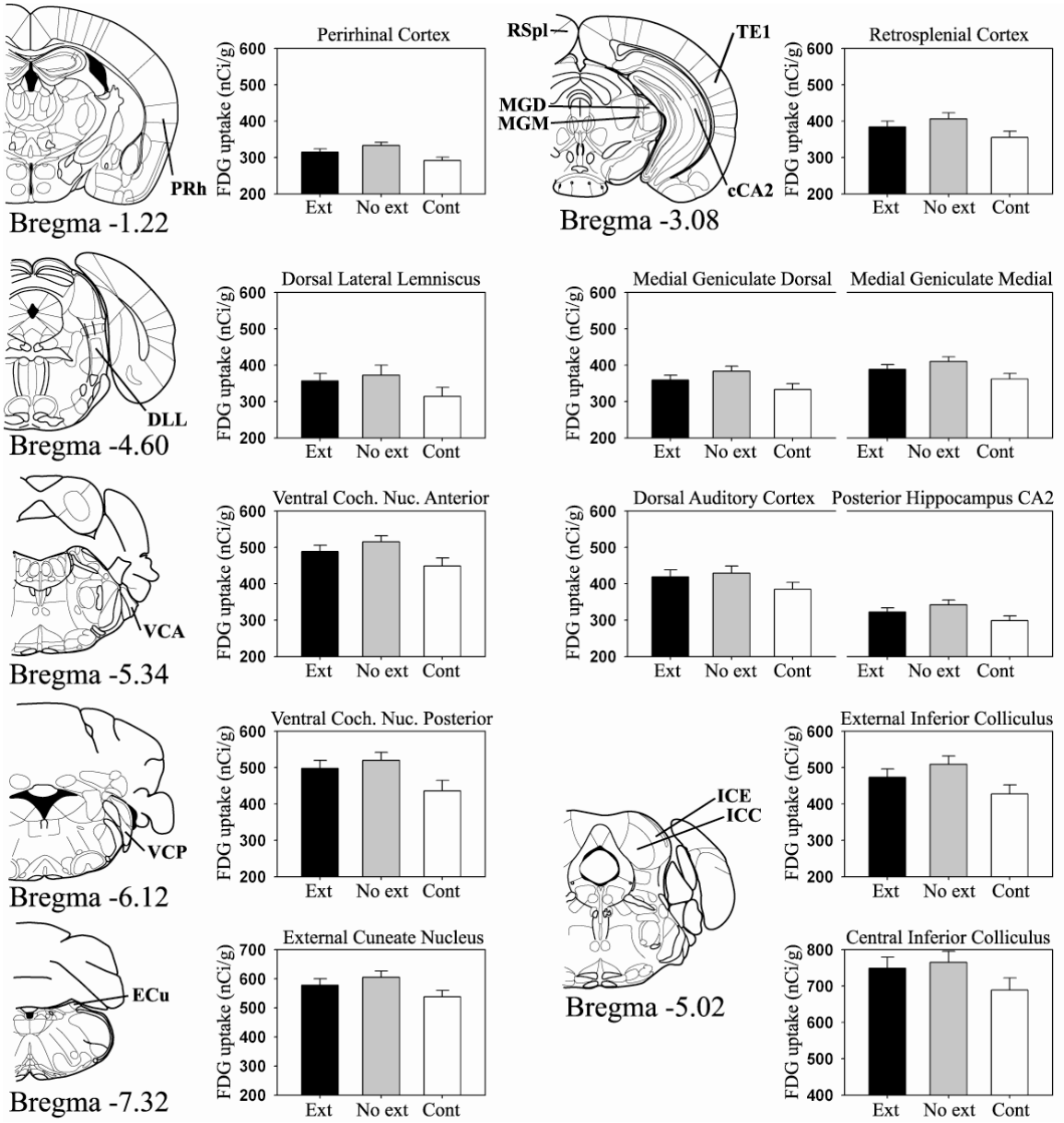


Figure 2.7: Regions of mouse brain showing increased FDG uptake in the non-extinction group, relative to pseudorandom (ANCOVA, $p < 0.01$). Means +/- SEs are expressed as nanocuries of isotope incorporation per gram brain tissue, with white matter readings from optic tract as covariates. Abbreviations: PRh, perirhinalcortex; DLL, dorsal lateral lemniscal nucleus; VCA, anterior ventral cochlear nucleus; VCP, posterior ventralcochlear nucleus; ECu, external cuneate nucleus; RSpl, retrosplenial cortex; MGD, medial geniculate nucleus, dorsal; MGM, medial geniculate nucleus, medial; TE1, dorsal auditory cortex; cCA2, posterior hippocampus CA2; ICE, inferior colliculus, external nucleus; ICC, inferior colliculus, central nucleus.



3) Non-extinction group greater than extinction group.

(Figure 2.8) The nucleus of the trapezoid body (TBN) in the non-extinction group showed 10% higher activity than in the extinction group, which displayed activity similar to the pseudorandom group, and the difference between the non-extinction and pseudorandom groups approached statistical significance. Given the consistent excitatory effects for the other auditory system regions, and the similar pattern exhibited in the TBN, this region may be showing a weaker excitatory effect.

The central amygdala and deep entorhinal cortex also showed significant increases (23-29%) in the non-extinction group relative to the extinction group, but neither group was significantly different from the nonassociative pseudorandom group. Relative to the pseudorandom group, the trends were for lower activity in the extinction group and higher activity in the non-extinction group, which may reflect nonassociative influences on the CER.

Extinction and non-extinction groups both greater than pseudorandom group.

(Figure 2.9) In spite of the different CER expressed in the non-extinction and extinction groups, their common acquisition training resulted in similar tone-evoked effects in the CA3 hippocampus, subiculum and pre-subiculum, seen as 10-18% increases relative to the pseudorandom group. These effects cannot be due to CER expression at the time of FDG uptake, because while the non-extinction group demonstrated freezing behavior, the extinction group did not. The simplest explanation is that acquisition training resulted in these changes due to the original tone-shock association. The medial geniculate, which also showed significant increases (10-12%) in both conditioned groups, might have entered into this association, contributing the auditory component of the CS-US savings.

Figure 2.8: Regions of mouse brain showing increased FDG uptake in the non-extinction group, relative to extinction group (ANCOVA, $p < 0.01$). Means +/- SEs are expressed as nanocuries of isotope incorporation per gram brain tissue, with white matter readings from optic tract as covariates. Abbreviations: TBN, trapezoid body nucleus; CeA, central amygdala nucleus; DEnt, deep entorhinal cortex.

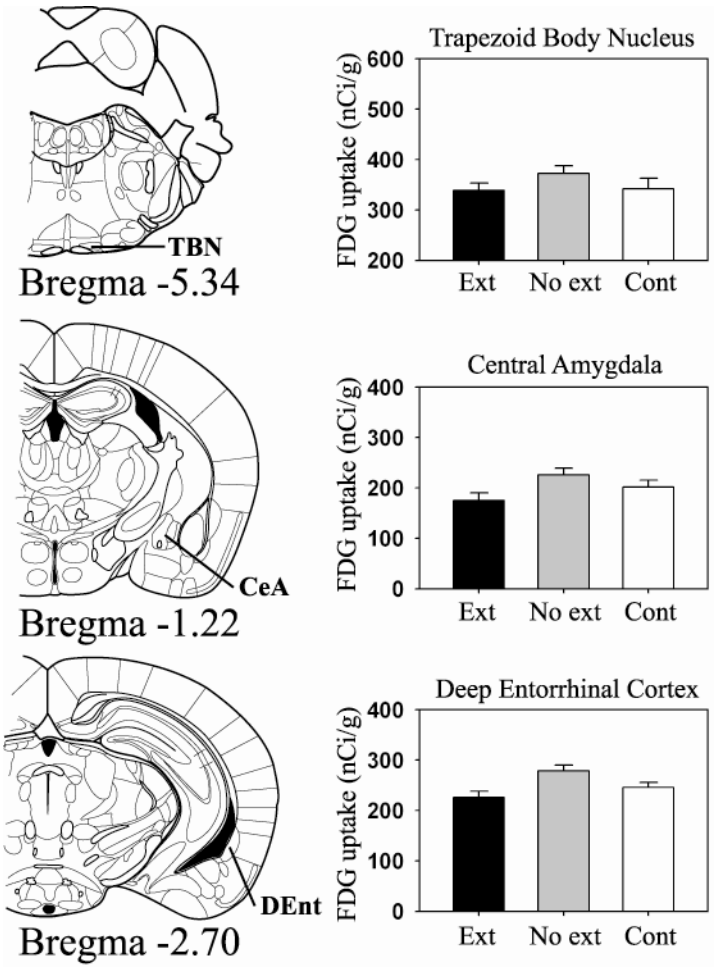
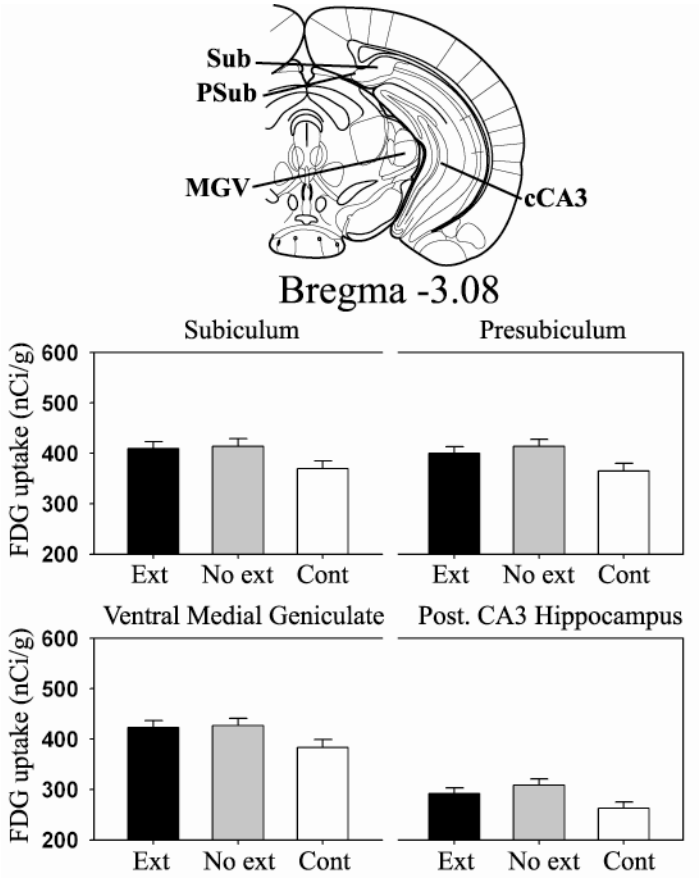


Figure 2.9: Regions of mouse brain showing increased FDG uptake in both extinction and non-extinction groups, relative to pseudorandom (ANCOVA, $p < 0.01$). Means +/- SEs are expressed as nanocuries of isotope incorporation per gram brain tissue, with white matter readings from optic tract as covariates.Abbreviations: Sub, subiculum; PSub, presubiculum; MGV, medial geniculate nucleus, ventral; cCA3, posterior hippocampus CA3.

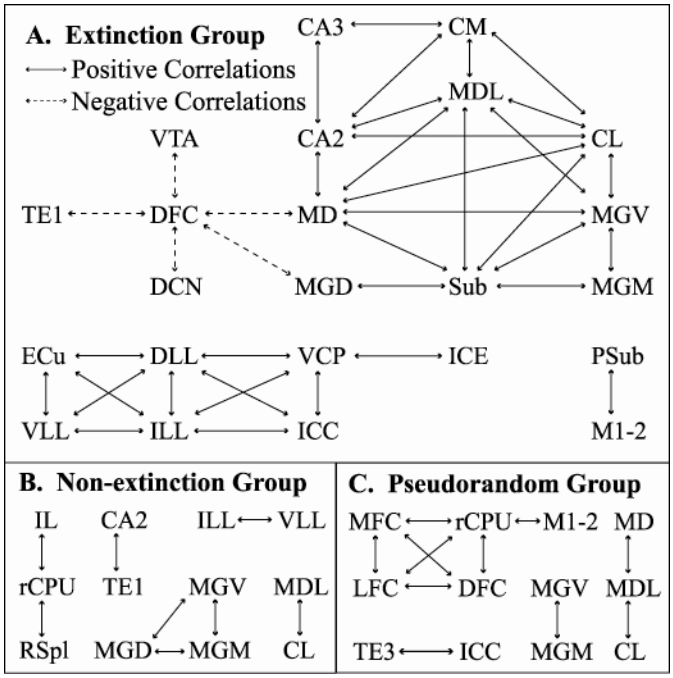


Inter-regional within-group correlations

A striking finding about the inter-regional correlations of FDG uptake activity was the large number of reliably significant correlations among regions within the extinction group, far more than were found in either the non-extinction or pseudorandom groups. Of 50 pairwise correlations which were found to be reliably significant at $p < 0.01$ after the conservative jackknife procedure, 37 of these were found in the extinction group, with 5 of those as 0.99 negative correlations between dorsal frontal cortex and other structures. These patterns of interactivity are illustrated in Figure 2.10. Significant correlation coefficients for each pairwise combination of regions showing significant group effects are listed in appendix Table 6.3.

In the extinction group, dorsal frontal cortex (DFC) activity was inversely correlated with the VTA, MD thalamus, and auditory regions (DCN, MGD, TE1). These strong negative correlations provided compelling evidence for a negative relationship between the prefrontal cortex and circuits linked to tone-evoked excitation of the conditioned response. The prefrontal cortex showed high co-linearity among some of its regions indicating a unified general pattern of activation and similar influences to other regions. Although a similar pattern of interactivity was found for DFC-LFC-MFC, only the stronger negative correlations between DFC and other regions reached significance in the extinction group. Two other functional circuits were formed in the extinction group, a higher-level circuit between medial thalamic and hippocampal regions and a lower-level circuit between auditory and somatosensory brainstem nuclei. In the higher circuit, the MD and MGD were functionally connected to a highly interactive thalamic-hippocampal network combining medial thalamic nuclei (MDL, CM, CL, MGV, MGM) and hippocampal regions (CA2, CA3, Sub). A brainstem circuit was also formed between CS

Figure 2.10: Pairwise inter-regional activity correlations by group. Solid arrows indicate significant positive correlations in metabolic activity between two regions; Dashed arrows indicate significant negative correlations ($p<0.01$). The extensive functional coupling in the extinction group implies the existence of a network of thalamic, hippocampal and auditory regions, with frontal cortex showing negative correlations with other regions.



and US relay pathways, comprising the lower auditory nuclei (VCP, DLL, ILL, VLL, ICC, ICE) and a somatosensory relay nucleus (ECu).

In the non-extinction group, a smaller number of reliably significant correlations were found between infralimbic cortex, rostral caudate-putamen, and retrosplenial cortex, as well as auditory thalamocortical system/ hippocampal correlations. The pseudorandom group showed significant positive relationships among the frontal regions and more coupling between frontal regions and cortico-striatal motor regions (rCPU, M1-2). In both the non-extinction and pseudorandom groups, the rostral caudate-putamen showed significant correlations with other regions. These correlations were not observed in the extinction group.

As a result of the jackknife procedure, some high correlations were not reliably significant at $p < 0.01$. For example, the value of the correlation between dorsal frontal and lateral frontal cortex (DFC-LFC) was 0.95-0.96 for all three groups, but only the pseudorandom group remained significant throughout all the jackknife iterations. Dropping subject 12 from the extinction group resulted in $p = 0.039$, while dropping subject 16 from the non-extinction group gave $p = 0.011$. While many more high correlation coefficients could reach a less conservative probability level, the same within-group patterns of interactivity were found. Rather than focusing on particular correlation coefficients, the inter-regional covariance approach emphasized how the pattern of relationships among many regions and systems was manifested in each group.

Brain-behavior correlations

Correlations between brain activity and extinction behavior for the five regions showing significant ($p < 0.05$) correlations are presented in Table 2.3. This analysis confirmed that each of the more activated regions in the extinction group showed positive

Table 2.3: Significant brain-behavior correlations between FDG activity and extinction index.

Region	Correlation (r value)
Medial frontal cortex (MFC)	0.99**
Dorsal frontal cortex (DFC)	0.61*
Lateral frontal cortex (LFC)	0.82**
Infralimbic cortex (IL)	0.64**
Rostral caudate-putamen (rCPU)	0.48*

**Significant at $p<0.01$; *Significant at $p<0.05$.

correlations between regional activation and extinction of the CER. The MFC in particular showed a 0.99 correlation between the extinction retention index and cortical activity ($p < 0.01$). The subjects with higher medial frontal cortex activity were more successful at inhibiting the CER.

DISCUSSION

In an intact mammalian brain, prefrontal cortex activation and its negative interactions with extensive networks of medial thalamic, auditory and hippocampal regions underlie the retention of extinction. Behavioral phenomena such as spontaneous recovery suggest that CS-US associative effects are not eliminated after extinction. However, many neural studies assume that extinction is simply the reversal of acquisition. For example, in the mollusk Hermissenda, Richards et al. (1984) concluded that extinction results from a reversal of the acquisition process, in terms of behavior and electrophysiology. While this may be the extinction mechanism in simple organisms like invertebrates, animals with complex brains have more complex mechanisms of extinction that cannot be reduced to a simple cellular event.

Neural activity differences between extinction, non-extinction and pseudorandom groups of mice might indicate the neural mechanisms involved in inhibition of the conditioned response. Since the extinction group as well as the pseudorandom group did not show a CER during FDG uptake sampling of brain activity, neural effects unique to the extinction group cannot be discounted simply as reflecting differences in CER performance. The lack of tone-evoked conditioned effects in the basal and lateral amygdala has been a consistent negative finding in every FDG study of conditioning for the past twenty years. Some regions might be involved at the beginning of conditioning but not after several days of training, when we tested the tone-evoked effects.

A strong argument can be made for a crucial role of the prefrontal cortex in the retention of CER inhibition after extinction. The lesion literature, however, is not conclusive. First, lesions are often made before acquisition training, which would interfere with normal brain interactions in acquisition and extinction. For example, the frontal-auditory correlation (DFC-DCN) was modified from positive to negative in the acquisition and extinction groups (0.52 to –0.99). Frontal cortex lesions would compromise this interaction during both acquisition and extinction. Second, lesions assume that the mechanism of extinction is localized to one brain region or pathway. This is not the case, as shown by the extensive network of interactions between brain regions in the extinction group (Figure 2.10). Lesions of the rat ventromedial prefrontal cortex resulted in across-days extinction deficits in studies by Morgan et al. (1993) and Quirk et al. (2000) but not in Gewirtz et al. (1997). Morgan and LeDoux (1995) performed electrolytic lesions of the rat dorsomedial prefrontal cortex before Pavlovian conditioning, and found that rats increased their freezing during both acquisition and extinction. Vouimba, Garcia, Baudry, & Thompson (2000) performed electrolytic lesions of the mouse dorsomedial prefrontal cortex after acquisition of Pavlovian conditioning, and found no effect on extinction. The assumption that there is one region or pathway responsible for extinction is too simplistic considering that in intact brains the extinguished tone produced activational effects in many regions (Figure 2.3) and that there were over thirty different inter-regional interactions in the extinction group that were not observed in the acquisition and pseudorandom groups (Figure 2.10).

Milad et al. (2002) provided electrophysiological evidence for the involvement of the rat infralimbic cortex in the retention of extinction. Single unit recordings showed enhanced firing rates in infralimbic cortex but not in medial orbital or prelimbic cortex. Unit responses to the tone were stronger in rats showing more extinction of freezing.

Furthermore, electrical stimulation of the infralimbic cortex led to less freezing during extinction. The electrical stimulation of projections from MD to prefrontal cortex can also modify extinction of conditioned freezing (Herry et al., 1999; Herry et al., 2002). These findings are consistent with our FDG results. The mouse infralimbic cortex showed the largest increase in FDG uptake (23%) among the regions with significantly elevated activity in the extinction group. However, orbital and prelimbic cortex showed no significant group differences. Infralimbic activity was also correlated with our behavioral extinction index. Extinction effects, however, were not limited to the infralimbic cortex.

We found that medial, dorsal, and lateral regions of the prefrontal cortex, located anterior and dorsal to infralimbic cortex, showed higher correlations between their activity and the extinction index (Table 2.3), and they satisfied our criterion of showing differences as compared to both non-extinction and pseudorandom controls. The medial, dorsal and lateral frontal regions are labeled as Cg1, M2 and M1, respectively, in the Paxinos and Franklin (2001) atlas. Our medial frontal cortex region (Figure 2.4) is a neocortical region with six layers that needs to be distinguished from the histologically different cingulate cortex located more posterior, also labeled Cg1 by Paxinos and Franklin (2001). DFC and LFC are labeled as M2 and M1, respectively, in the Paxinos and Franklin atlas at this level (2.22 mm anterior to Bregma) and at much more posterior levels (1.22 mm posterior to Bregma). We cannot use the M1-M2 labels at all these levels because while there is evidence that posterior M1-M2 are motor regions, there is no such purely motor evidence for our much more anterior dorsolateral frontal regions. M1-M2 showed no differences between extinction and non-extinction groups, and our results support that DFC and LFC are linked to extinction rather than to purely motor effects.

There were also large-scale networks of interactions between dorsal frontal, medial thalamic, hippocampal and auditory regions in the extinction group, which may reflect an inhibitory relationship between frontal cortex and auditory and limbic networks with CS-US associative effects. This interpretation is consistent with human neuroimaging studies of tone conditioning. For example, Molchan, Sunderland, McIntosh, Herscovitch & Schreurs (1994) found an increase in frontal cortex blood flow with extinction, whereas auditory and medial temporal regions showed increases during acquisition. Schreurs, Bahro, Molchan, Sunderland & McIntosh (2001) examined the interactions of prefrontal cortex during acquisition and extinction of tone-conditioned eyeblink in young and old people using blood flow data. Consistent with our FDG findings in mice, humans showed greater activity in the prefrontal cortex during extinction retention. Moreover, after extinction the prefrontal cortex interacted extensively with other regions that were activated during acquisition, including negative correlations with auditory regions (superior temporal areas 42, 22) and limbic regions (hippocampus, perihippocampal area). Older subjects with impaired tone conditioning did not show these interactions. Admittedly, homologies between human and rodent brains are difficult to make and the spatial resolution in the human studies was very limited as compared to our FDG mapping. Nevertheless the general pattern of brain effects produced by an extinguished tone is essentially the same in mice and men.

The prefrontal cortex is also involved in other behavioral inhibitory phenomena, such as the blocking of tone conditioning (Jones et al., 2001b) and the partial reinforcement extinction effect (Nair et al., 2001b; 2001a). Our FDG study of the extinction of an instrumental response in infant rats found significant interactions between medial prefrontal, orbitofrontal, and anterior cingulate cortices, but only in the older pups, which extinguished faster than the younger pups. Extensive functional

coupling was found in the older group during extinction but not in handled controls, suggesting that the functional network involving frontal cortex was present as a result of their extinction training. McIntosh, Rajah, & Lobaugh (1999) described a PET study in which human subjects showed progressively greater prefrontal activity to a tone CS- than another tone CS+. The better they were at inhibiting their response to the CS-, the more blood was routed to their prefrontal cortex. This form of behavioral inhibition is similar to the extinction paradigm, in which a tone-evoked CER is being suppressed.

Our findings also provided evidence that some auditory system changes produced by acquisition are present after extinction. Most auditory system activation is linked to tone-signaled CERs, as in our previous FDG mapping studies comparing tone-conditioned CER excitation and inhibition (McIntosh & Gonzalez-Lima, 1994). Associative effects in the auditory system are not observed in the pseudorandom group, in which the tone was not associated with the US. Enhanced activity in the ventral medial geniculate nucleus in non-extinction and extinction groups suggests that extinction does not involve unlearning of excitatory CS-US neural associations in some auditory structures.

The activational effects in the hippocampus, across both extinction and non-extinction groups, is further evidence that the previously acquired tone-shock association is still present in the extinction group, even though the CER is extinguished. Interestingly, this lingering excitatory CS-US effect was not found in another form of CER inhibition called differential inhibition, where the tone is never paired with the footshock, and no excitatory tone-shock association can be formed. In the case of differential inhibition, hippocampal and septal areas exhibited decreased FDG uptake to the tone inhibitor as compared to a pseudorandom group (Jones et al., 2001a). Therefore, excitatory CS-US associative effects in certain auditory and hippocampal regions are not

destroyed with extinction, and the brain effects of an extinguished tone are not the same as those of a tone inhibitor.

In conclusion, the findings suggest that prefrontal activation inhibits the associative components of the tone-evoked conditioned response via its negative interactions with auditory and hippocampal networks. They also support Pavlov's (1927) ideas of extinction, namely that the original CS-US associative effects remain partially intact and that inhibitory cortical circuits are formed to reduce the CS-evoked conditioned response.

Chapter 3: Extinction Deficit in Congenitally Helpless Rats

INTRODUCTION

Helplessness is a behavioral state of passivity accompanied by feelings of uncontrollability and is a common component of several comorbid psychiatric disorders, most notably major depression and post-traumatic stress disorder (PTSD). Inescapable electric shock prevents animals from subsequently learning an escape response, a phenomenon called learned helplessness (Overmier et al., 1967). The congenitally helpless strain of rats was developed by selectively breeding animals that showed greater susceptibility to learned helplessness (Henn et al., 1985; Henn et al., 1994). The helpless strain exhibits an altered behavioral phenotype, which predisposes them to develop helpless behavior in response to stress (Vollmayr et al., 2004).

We have found extensive alterations in regional brain metabolism in congenitally helpless rats (Shumake et al., 2000; Shumake et al., 2001; Shumake et al., 2002; Shumake & Gonzalez-Lima, 2003b; Shumake et al., 2003a; Shumake et al., 2004), including prefrontal cortex regions related to behavioral extinction (Barrett et al., 2003; Nair et al., 2001b; Nair et al., 2001a). One prediction based on these data is that congenitally helpless subjects will show impaired extinction following Pavlovian fear conditioning because they have impaired metabolism in prefrontal cortical regions mediating extinction (Gonzalez-Lima & Bruchey, 2004). This could lead to persistent fear-related conditioned responses to a Pavlovian conditioned tone, which may be more resistant to extinction than in normal control rats.

The use of the congenitally helpless genetic strain as a model for susceptibility to depression (Henn et al., 1994) and PTSD (King, Abend, & Edwards, 2001) has been reviewed recently (Shumake et al., 2003b). Extinction deficits have been implicated in

PTSD (Charney, Deutch, Krystal, Southwick, & Davis, 1993), and PTSD patients show reduced extinction of aversively conditioned responses (Peri, Ben Shakhar, Orr, & Shalev, 2000). As in PTSD, congenitally helpless rats may be predisposed to form traumatic memories which are persistently expressed in the absence of an aversive stimulus. Therefore, the primary objective of the present study was to test the hypothesis that congenitally helpless rats show resistance to extinction of a fear-evoking memory.

Congenitally helpless rats may also show gender differences in extinction, given the increased risk of PTSD found in human females (American Psychiatric Association, 2000). Hence our second objective was to compare the extinction behavior of female and male rats of the congenitally helpless strain. The first and second objectives were pursued in an experiment using a Pavlovian tone-footshock fear conditioning paradigm to induce the formation of an aversive emotional memory. The relative strength and persistence of this fear-evoking memory was measured in terms of tone-evoked freezing behavior and inhibition of motor activity, during extinction sessions and during probe trials in both acquisition and extinction contexts. The behavioral effects found and their implications for PTSD are considered in light of brain and behavioral studies of extinction (Barrett et al., 2003; Nair et al., 2001b; Nair et al., 2001a; Barrett et al., 2004).

METHODS AND MATERIALS

Subjects

A total of 57 Sprague-Dawley rats were divided into four groups: 23 female congenitally helpless rats, 10 male congenitally helpless rats, 12 female control rats and 12 male control rats. Subjects weighed approximately 400 g for males and 300 g for females at the start of the experiment.

The congenitally helpless line, developed by Henn and Edwards (1994), were selectively bred based on their performance in an escape paradigm 24 hours after 20 minutes of 0.8 mA footshock distributed randomly within a 40 minute session. Animals with more than 10 failures out of 15 trials were considered helpless, and subjects meeting this criterion were mated over subsequent generations, avoiding sibling crosses. The congenitally helpless subjects used in this study were descendents of this line bred in our laboratory and were not themselves tested for susceptibility to learned helplessness; however, over 95% of the offspring typically show the phenotype. The 24 control Sprague-Dawley rats were obtained from Harlan (Houston, Texas). In both groups, females were housed two-to-three per cage and males were singly housed, in order to conform to NIH guidelines regarding amount of cage space required based on animal weight. (Some males were too large to be co-housed in our cages.) Subjects were housed under standard laboratory conditions with a 12 hr light/dark cycle and ad libitum access to food and water. Animal experimentation was approved by the University of Texas Institutional Animal Care and Use Committee.

Apparatus

Acquisition context

Pavlovian tone-shock acquisition training was performed in four conditioning chambers (30 x 25 x 20 cm) (Med Associates, St. Albans, VT) enclosed in sound-attenuated boxes illuminated by a red light. Two sides of each chamber were aluminum, with clear Plexiglas for the front, back, and top. Tones were generated by a Wavetek Sweep/Modulation Generator (Wavetek, San Diego, CA) and presented through speakers mounted at the top of each chamber. The acoustic conditioned stimulus (CS) was a

frequency-modulated tone of 1-2 kHz, 2 sweeps/sec, 15 sec in duration, with an intensity of 68 dB, measured at the center of the floor of the chamber. The unconditioned stimulus (US) was a footshock of 0.5 mA, 0.75 sec in duration, delivered through metal bars separated by 1.2 cm forming the floor of the chamber, which was wired to shock generators (Med Associates). Stimulus presentations were controlled by computer programs, created using the MED-PC for Windows programming language (Med Associates). A Bioclean solution (Stanbio Laboratory, Boerne, TX) was placed in the tray beneath the chamber to provide a distinct olfactory cue.

Extinction context

Extinction training occurred in a different context: two open-field activity boxes (43 x 43 x 30.5 cm) (Med Associates). Each arena had a fiberglass bottom, clear Plexiglas for the four sides, and an open top. Horizontal activity was detected by arrays of infrared motion detectors (16 x 16, 1 inch apart), with two arrays 1 cm above the floor of the chamber. Rearings were detected with a vertical-axis array positioned 13 cm above the surface of the floor for female subjects, and 15.5 cm above the surface for male subjects, to ensure that only those rearing movements in which the subject's forepaws left the ground would register as rearing counts. The chambers were controlled by the Activity Monitor program, version 5.10 (Med Associates), which records various parameters related to the subject's locomotion, resting and rearing behaviors. The 1-2 kHz tone CS was digitally recorded from the tone generator and presented through a computer speaker above the open fields, and measured 68 dB at the center of the floor of each open field.

Behavioral Training

Conditioned behavior

Two measures were used as indices of tone-evoked fear: an observer-scored freezing measure and a computer-scored immobility measure. Freezing was operationally defined as the subject having all four feet on the floor, with minimal head movements and shallow, rapid breathing for 3 sec, for a maximum freezing score of 5 for complete immobility during the 15-sec tone CS presentation. Immobility time consisted of 50 msec intervals in which the subject did not incur any new beam breaks in the open field apparatus. A correlational analysis revealed a high reliability between the two measures, $r = .81$, $p < .01$. The computer does not distinguish between resting and freezing and may also register some aspects of grooming as immobility. These two factors likely account for the imperfect relationship between freezing and immobility, and should be considered when comparing the results for each measure.

Experimental design

Both male and female subjects were handled every day for at least 7 days before the start of training. During this first week, each subject was habituated to the acquisition context in the absence of tones or shocks each day for 1 hour. Vaginal smears were performed on female subjects to determine estrus cycle phase for each subject before training began. An estrus cycle of 4 days was confirmed for all female subjects. Training of female subjects was staggered according to estrus cycle, such that the first day of acquisition training occurred while each female subject was in estrus. (Diestrus females received up to 3 additional days of habituation / handling.) The training procedure is presented in Table 3.1.

Table 3.1: Experimental design of congenitally helpless rat experiment

Experiment 1	Day 1	Days 2 - 3	Day 9	Day 10
Session	Acquisition	Extinction	Probe-Acq	Probe-Ext
Context	Context A	Context B	Context A	Context B
Stimuli	4 T → S	30 T / Day	4 T	4 T

T, 1-2 kHz 68 dB 15 sec tone CS; S, 0.5 mA 0.75 sec footshock US.

Phase I

For acquisition training, all subjects were placed in the conditioning chambers (context A) and received 4 tone-shock pairings over 15 minutes, presented with pseudorandom intertrial intervals which averaged 3 minutes each. Each 15-sec tone CS co-terminated with the footshock US.

Phase II

Extinction training occurred over two consecutive days while female subjects were in the diestrus phase, to control for any hyperactivity during estrus (Birke & Archer, 1975) which might interfere with conditioned freezing measures in the open-field. All subjects were placed in the open field (context B), and 30 tones were presented over 60 min, with 2 minutes between each tone onset, starting one minute after the open-field session began. Activity was recorded by the computer and parsed into 15-sec intervals which were synchronized to 15-sec tone CS presentations, which allowed for specific analysis of tone-CS-evoked immobility during the CS periods alone. The first minute in the open-field context served as a baseline measure of activity, before the first tone CS was presented.

Probe trials

Post-extinction probe trials in the acquisition context occurred one week later, when female subjects were in estrus, and served to measure the renewal effect. Each subject was returned to the conditioning chamber (context A) and presented with four 15-sec tones (in the absence of footshock) over 10 min, with 2 min intervals between each tone onset. Post-extinction probe trials (4 tone CSs as described above) in the extinction context occurred the following day, when female subjects were in diestrus, and served to measure retention of extinction learning. Thus, the hormonal context of each female

subject's estrus phase during training was matched to the physical training context for probe trials in both the acquisition and extinction contexts.

Statistical Analysis

The SPSS 11.5 for Windows statistical software package was used for statistical analysis. All data were analyzed with analyses of variance (ANOVAs), and p-values are reported as Huynh-Feldt corrected values, with an alpha value of .05 regarded as significant. When warranted, simple-effects tests of significant interactions were performed and adjusted by Bonferroni correction.

RESULTS

Helpless and control subjects showed similar baseline open-field activity and conditioned freezing prior to extinction training. In contrast, they showed dramatic group differences during extinction, with the helpless rats of both sexes exhibiting a large extinction deficit, in terms of both performance and retention.

Baseline Activity

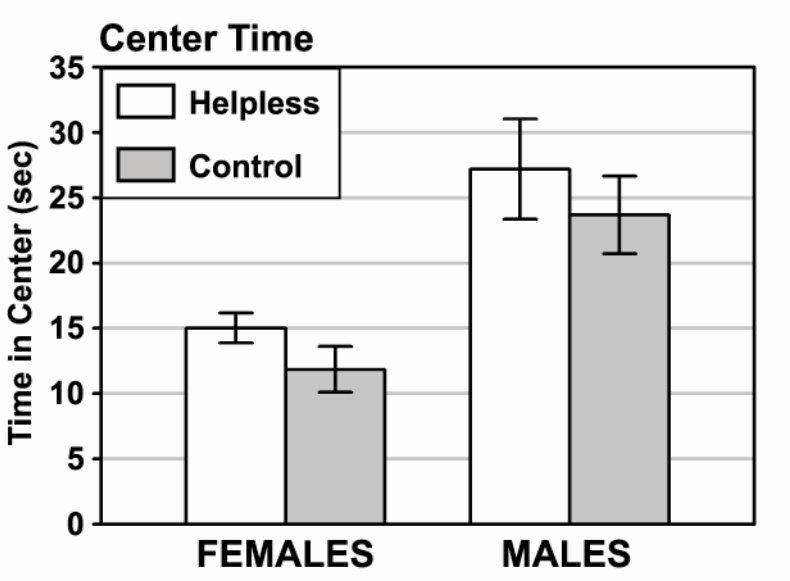
Locomotion during the first minute of extinction session 1 (prior to the onset of the first tone CS) showed no group differences in any of the parameters analyzed, $F(1,53) < 2.4$, but there were significant sex differences in ambulation, rearing, immobility, and thigmotaxis, $F(1,53) > 4$, $p < .05$. Females in both groups spent significantly more time ambulating and rearing and less time resting than males (Table 3.2), while males spent more time in the center (Figure 3.1). The absence of significant group differences on these baseline measures indicated that differences observed in extinction behavior cannot be attributed to baseline strain differences in general motor activity or fearfulness.

Table 3.2: Motor activity (sec) during one minute of baseline open field (pre-CS1).

	FEMALES		MALES		P-Values (ANOVA)		
	Helpless Mean ± s.e.	Control Mean ± s.e.	Helpless Mean ± s.e.	Control Mean ± s.e.	Effect of Group	Effect of Sex	Group by Sex
Baseline (1 min)							
Ambulatory Time	9.7 ± 0.8	9.6 ± 1.2	4.1 ± 0.8	5.9 ± 1.0	p = 0.447	p < 0.001*	p = 0.350
Stereotypic Time	18.8 ± 0.7	18.9 ± 0.8	17.3 ± 1.0	18.5 ± 0.9	p = 0.423	p = 0.271	p = 0.537
Immobility Time	15.6 ± 1.6	20.8 ± 2.7	30.0 ± 2.9	26.4 ± 3.1	p = 0.758	p < 0.001*	p = 0.081
Rearing Time	14.3 ± 1.5	9.2 ± 1.2	8.2 ± 1.5	8.2 ± 1.5	p = 0.129	p = 0.034*	p = 0.124

* $p < 0.05$

Figure 3.1: Measures of thigmotaxis (mean ± standard error bars) in congenitally helpless and control rats. Activity during the first minute in the open field tested for group differences in baseline anxiety, prior to the first tone onset. There were no significant group differences in center time. The center region was defined as 38% of the total area as in our recent study (Gonzalez-Lima and Bruchey, 2004).



A similar analysis of motor activity during the 1-minute interval after the first tone CS of extinction session 1 is presented in Table 3.3. From pre-CS1 to post-CS1, increases in immobility time were found in every group and sex, with concomitant decreases in ambulatory, stereotypic and rearing time. The only significant finding during post-CS1 was a sex difference in one motor parameter (stereotypic time). There were no significant group differences in ambulatory, stereotypic, immobility or rearing times for the post-CS1 interval. This result demonstrates that the freezing CR is specific to the tone CS in congenitally helpless rats. While this strain is vulnerable to spontaneous helpless behavior after footshock, it is unlikely that the immobility seen during CS presentations is the result of non-associative US sensitization effects carried over from acquisition. Freezing behavior resulting from US sensitization effects would likely extend into no-tone intervals, yet no group differences in immobility time or any other motor parameter was found in either pre-CS1 or post-CS1 intervals.

Short-term Extinction

Short-term extinction was assessed by comparing changes in freezing and immobility within each hour and across both days of extinction training. The data were evaluated with a 2 × 2 × 2 × 6 (Group × Sex × Session × Time) repeated measures ANOVA, with session and time serving as repeated measures. Behavioral measures from each 15-sec tone CS presentation were averaged into 6 bins representing 5 tone presentations every 10 minutes (Figure 3.2).

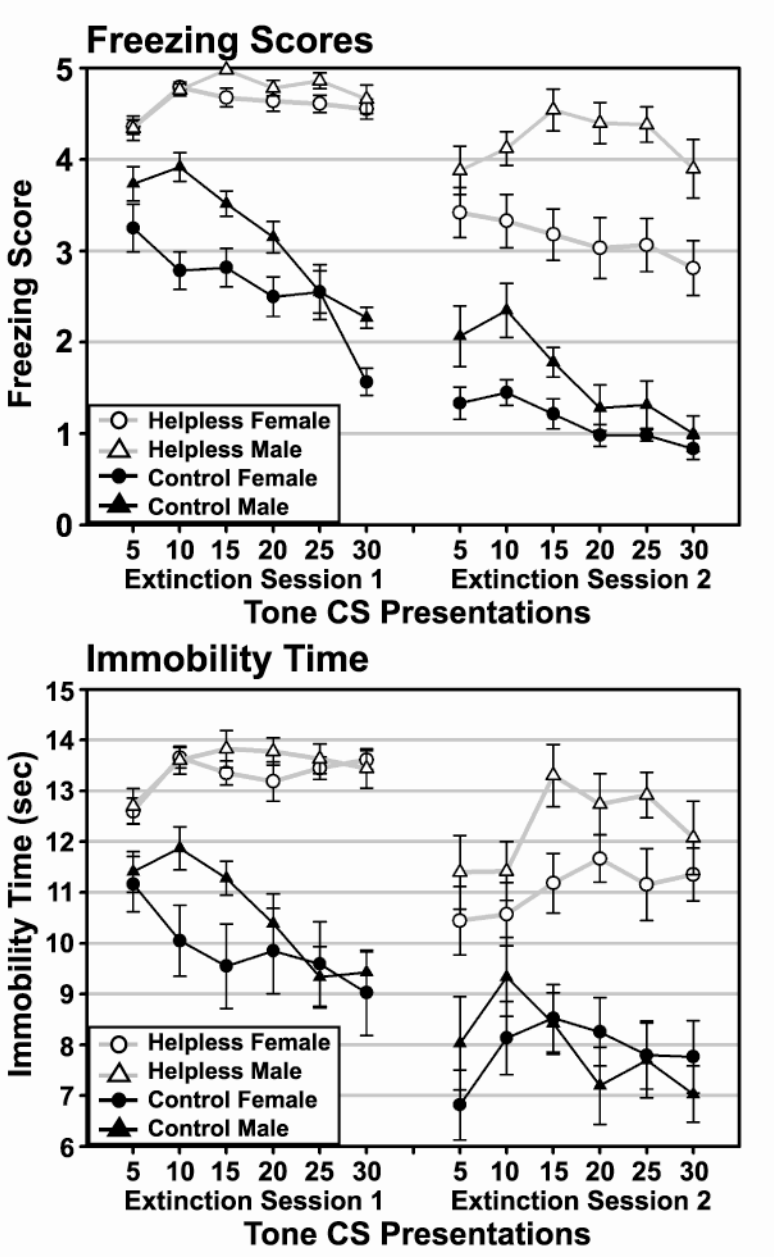
A significant three-way interaction (Group x Session x Time) was obtained for freezing scores, $F(5,245) = 3.618$, $p < .01$. The interaction for freezing indicated that while control rats showed similar rates of extinction in both sessions, congenitally helpless rats did not show any evidence of extinction in the first session and only began to extinguish in the second session. Related to this, a significant two-way interaction

Table 3.3: Motor activity (sec) during one minute after first extinction tone (post-CS1).

	FEMALES		MALES		P-Values (ANOVA)		
	Helpless Mean ± s.e.	**Control** Mean ± s.e.	**Helpless** Mean ± s.e.	**Control** Mean ± s.e.	**Effect of Group**	**Effect of Sex**	**Group by Sex**
Baseline (1 min)							
Ambulatory Time	3.7 ± 0.7	3.3 ± 1.1	1.0 ± 1.0	3.8 ± 1.8	p = 0.336	p = 0.352	p = 0.168
Stereotypic Time	14.1 ± 1.3	12.2 ± 1.6	9.0 ± 1.8	8.8 ± 2.0	p = 0.555	p = 0.017*	p = 0.622
Immobility Time	36.2 ± 2.8	38.2 ± 4.5	45.5 ± 4.6	41.8 ± 6.0	p = 0.849	p = 0.147	p = 0.517
Rearing Time	6.1 ± 1.1	5.4 ± 2.2	4.5 ± 2.8	4.8 ± 2.1	p = 0.931	p = 0.567	p = 0.782

* $p < 0.05$

Figure 3.2: Extinction curves (mean ± standard error bars). The conditioned response during extinction sessions 1 and 2 was measured in terms of freezing behavior (top) and immobility time (bottom). Each 1-hour extinction session consisted of 1 tone CS every 2 min, averaged by 5 tones for each 10-min bin for repeated measures ANOVA. Significant differences ($p < .01$) between helpless and control groups were found at every time point in both sessions.



(Group × Time) was found for immobility time, $F(5,265) = 5.5$, $p < .001$, indicating that, on this measure, congenitally helpless rats did not show extinction within either session.

A second significant three-way interaction (Group x Sex x Time) was obtained for freezing, $F(5,245) = 2.745$, $p < .05$, but not for immobility, $F(5,265) = 1.4$. The interaction indicated that, within sessions, helpless males and females showed similar decreases in freezing whereas control males showed a larger reduction in freezing than control females. However, a different pattern was observed between sessions, with helpless males showing a smaller reduction in freezing than helpless females and control males and females showing similar reductions in freezing. This was indicated by a significant three-way interaction (Group × Sex × Session) obtained for both freezing, $F(1,49) = 8.003$, $p < .01$, and immobility, $F(1,53) = 4.2$, $p < .05$.

While these interactions were all statistically significant, they are relatively trivial with respect to the simple comparison of helpless versus control rats. Tests of simple effects showed that the helpless group (males and females combined) displayed more freezing and immobility at every time point in both sessions ($F > 9$, $p < .01$).

Long-term Extinction

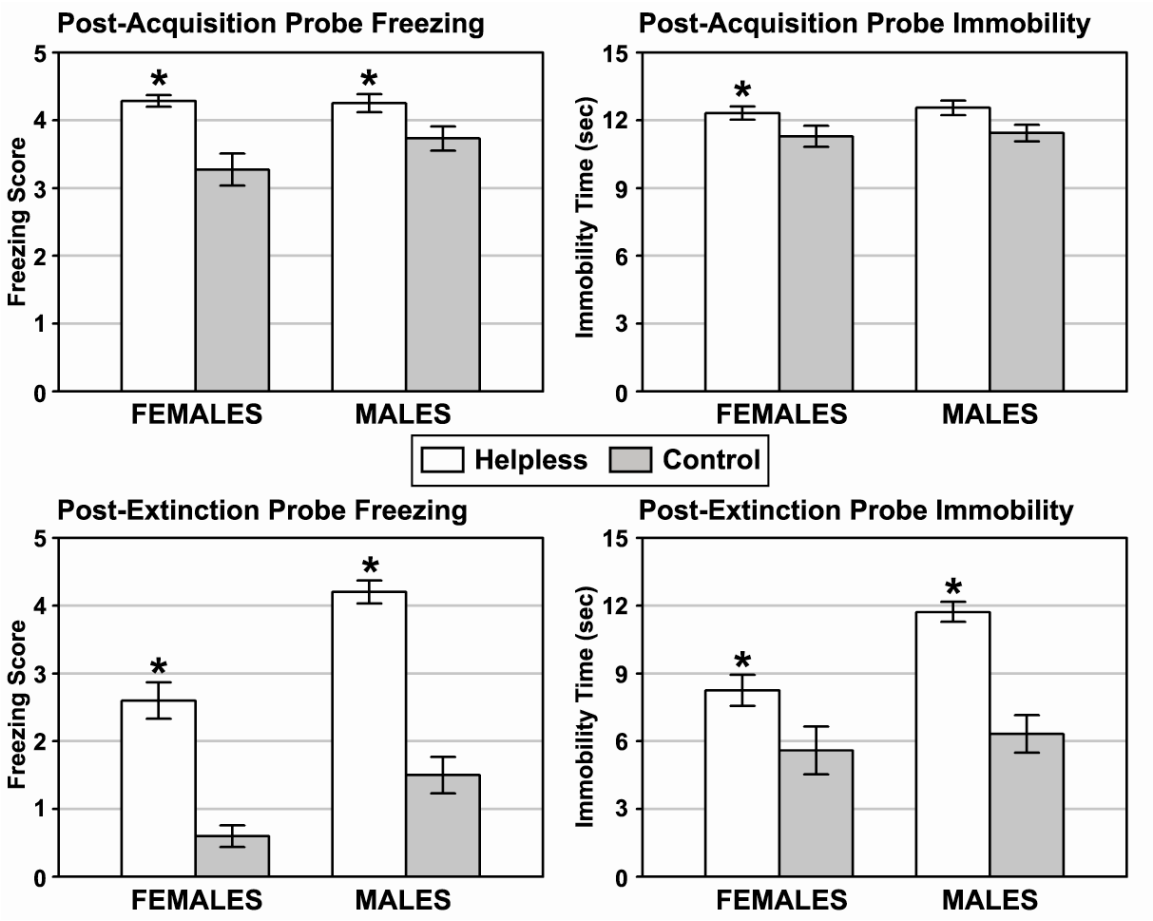
Long-term extinction was assessed by comparing freezing and immobility during the first four tones at the beginning of the extinction session (acquisition probe) with freezing and immobility during the four probe trials conducted one week after the end of extinction training (extinction probe). Data were analyzed with a 2 × 2 × 2 (Group × Sex × Probe) repeated measures ANOVA, with the averages of the four trials from the acquisition and extinction probes serving as repeated measures.

A significant three-way interaction (Group × Sex × Probe) was obtained for freezing, $F(1,53) = 5.8$, $p < .05$. For ease of interpretation, Figure 3.3 shows this

interaction as 2 two-way (Group × Sex) interactions, one for each probe session. The interaction indicated that, while control males and females and helpless females showed long-term reductions in freezing as a consequence of extinction training, 1) helpless males showed no such long-term extinction, and 2) helpless females showed less long-term extinction than control females. The immobility data showed the same pattern as the freezing scores, but the interaction was not significant, $F(1,53) = 3.2$.

Simple-effects tests of this interaction indicated that helpless rats showed significantly more freezing than controls during both the acquisition and extinction probes, $F(1,53) > 4.5$, $p < .05$, though this group difference was much larger for extinction than acquisition, and larger for females than males (Figure 3.3). Immobility time showed a similar pattern; however, the differences were smaller than those observed in freezing and failed to reach significance in the simple comparison of males in the acquisition probe, $F(1,53) = 3.7$, $p = .06$. These simple effects indicate that 1) differences observed following extinction are not simply a preservation of differences in acquisition, but rather reflect a difference in extinction rate; and 2) although helpless females showed less absolute freezing than helpless males, the helpless females appear more severely affected than helpless males when freezing is considered relative to same-sex controls. For example, in the extinction probe, helpless males showed 180% more freezing than control males, while helpless females showed 333% more freezing relative to control females.

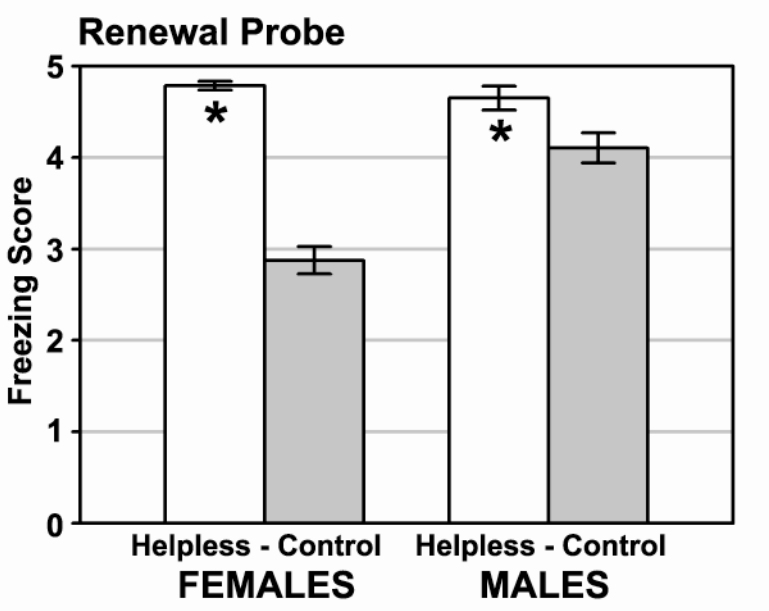
Figure 3.3: Probe trials (mean ± standard error bars) in the extinction context. The conditioned response was measured after acquisition (top) and after extinction (bottom) in terms of freezing behavior (left) and immobility time (right). Each probe test consisted of 4 tones in 10 min. * Significant group differences ($p < .05$).



Context Renewal

In order to assess the effects of context renewal on extinction, four tone probes were also given in the acquisition context (Figure 3.4). Freezing data from these probe trials were analyzed with a 2 × 2 × 2 (Group × Sex × Context) repeated measures ANOVA, with context (acquisition vs. extinction) and the averages of the four trials serving as repeated measures. A significant three-way (Group × Sex × Context) interaction was obtained, $F(1,53) = 14.6$, $p < .001$. Simple-effects tests of the interaction revealed that helpless females showed significantly more freezing relative to control females, $F(1,53) > 30$, $p < .001$, and this difference was equivalent in both contexts. Helpless males also showed significantly more freezing relative to control males, $F(1,53) > 9.4$, $p < .01$, but the difference was much greater in the extinction context than in the acquisition context. This appears to be due to both enhanced renewal in the male controls and to a ceiling effect in the helpless males, which were already freezing near maximum in the extinction context and at maximum in the acquisition context (Figures 3.3, 3.4).

Figure 3.4: Renewal probe trials (mean ± standard error bars) one week after extinction. Conditioned freezing to the tone in the acquisition context measured the renewal effect. The probe test consisted of 4 tones in 10 min. * Significant group differences ($p < .01$).



DISCUSSION

Congenitally helpless rats exhibited abnormally high freezing and immobility in response to a tone formerly predictive of electric shock, even after 60 presentations of the tone in the absence of shock. Helpless rats of both sexes failed to show the gradual decrement in freezing characteristic of the normal extinction curves seen in the control subjects. Instead, congenitally helpless rats continued to show significantly greater tone-evoked fear one week after extinction training, when tested in both the acquisition and extinction contexts.

The behavioral deficit seen in congenitally helpless rats cannot be explained by a general suppression of motor activity or by an overall increase in non-specific fearfulness, which can be indicated by thigmotaxis: time spent in the periphery vs. center of the open field (Treit & Fundytus, 1988). These alternative explanations are unlikely because there were no baseline group differences in open-field behavior prior to the onset of the fear-associated tones, in terms of motor activity and thigmotaxis.

Relevance to PTSD

Several convergent lines of evidence lead to the prediction that congenitally helpless rats would show deficits in Pavlovian extinction. Parallels between congenitally helpless rats and PTSD patients (King et al., 2001) can be found in similar patterns of altered behavior, glucocorticoid regulation, conditioned analgesia, and brain metabolism (Shumake et al., 2003b).

Behavior

This experiment demonstrated that congenitally helpless rats show deficits in both extinction performance and retention: an inherently neutral tone CS is capable of evoking maladaptive fearful responses in the helpless rats, even outside of the context in which they experienced the aversive shock US. PTSD is likewise characterized by the formation and persistence of traumatic memories across multiple contexts. PTSD patients have shown increases in autonomic arousal (heart rate, skin conductance) in response to aversive Pavlovian conditioning, and these conditioned responses are resistant to extinction (Orr et al., 2000; Peri et al., 2000).

Glucocorticoid regulation

PTSD patients may be characterized by impaired cortisol secretion (Yehuda, 1999), and Delahanty, Raimonde, & Spoonster (2000) showed that individuals demonstrating low cortisol levels after an acute trauma, such as motor vehicle accident, were at higher risk of subsequently developing PTSD symptoms. Congenitally helpless rats also show deficits in corticosterone secretion in response to stress (King et al., 2001). Adrenalectomized animals are more likely to show learned helplessness, and this susceptibility is reversed by corticosterone replacement therapy (Edwards, Harkins, Wright, & Henn, 1990). Corticosterone antagonism with metyrapone also interferes with Pavlovian extinction (Barrett et al., 2004). Thus, insufficient glucocorticoid signaling may be linked to PTSD, helplessness, and the fear extinction deficits observed in both.

Conditioned analgesia

King et al (2001) also found a 97% increase in stress-induced analgesia in congenitally helpless rats. This finding may be related to the 3-5 fold increase in mu-opiate receptors seen in congenitally helpless subjects (Henn et al., 1993). Opioid receptors have been implicated in extinction of Pavlovian fear conditioning, and infusions of the opioid receptor antagonist naloxone into the ventrolateral periaqueductal gray interfere with the extinction of Pavlovian fear conditioning (McNally, Pigg, & Weidemann, 2004). Elevated opioid-mediated analgesia, evoked by stress, has been implicated in PTSD patients (Charney et al., 1993), and naloxone can reverse stress-induced analgesia in Vietnam veterans with PTSD (Pitman, van der Kolk, Orr, & Greenberg, 1990). An elevated analgesic response to stress may also interfere with an adaptive behavioral response to the shift from acquisition to extinction, and the detection of non-reinforcement in extinction.

Brain metabolism

Our lab has extensively characterized the changes in brain regional metabolic activity found in congenitally helpless rats, in both adults (Shumake et al., 2000; Shumake et al., 2001; Shumake et al., 2002; Shumake et al., 2003a) and newborns (Shumake et al., 2004). Increased metabolism in the habenula (Shumake et al., 2003a), an epithalamic region implicated in several animal models of depression (Caldecott-Hazard, Mazziotta, & Phelps, 1988) and in human depression (Morris, Smith, Cowen, Friston, & Dolan, 1999), may be crucial to the persistence of traumatic memory, since lesions of the habenula result in no deficits in acquisition, but faster extinction rates (Brady & Nauta, 1955). Congenitally helpless rats also showed increased metabolism in the paraventricular hypothalamus (Shumake et al., 2001), which may be related to the

hypothalamic-pituitary-adrenal axis disruption of glucocorticoid regulation. Another region implicated in fear conditioning is the amygdala, which showed increased functional coupling with the anterior cingulate in congenitally helpless newborns (Shumake et al., 2004). Increased coupling between the amygdala and anterior cingulate was one of the few functional connections that differentiated PTSD patients from healthy trauma survivors (Gilboa et al., 2004) and may relate to the enhanced formation of traumatic memories.

Medial prefrontal cortex may be crucial for the inhibition of the conditioned response during Pavlovian and instrumental extinction (Barrett et al., 2003; Milad et al., 2002; Nair et al., 2001b; Nair et al., 2001a), and PTSD patients have shown decreased activity in medial prefrontal cortex during extinction (Bremner et al., 1999). In newborn congenitally helpless rats, brainstem regions are uncoupled from networks of frontal and limbic regions (Shumake et al., 2004). This decoupling between brainstem and forebrain regions may be indicative of a developmental disorder in which brainstem regions are removed from the inhibition provided by the frontal cortex during successful Pavlovian extinction. Such a disruption could selectively impair Pavlovian extinction while leaving acquisition intact.

Sex differences

The sex differences in the incidence rates of PTSD in humans and helplessness in the congenital helpless rat line implicate an increased vulnerability in females. Female humans show a 2-3 fold higher incidence of PTSD (DSM-IV, 2000), and female helpless rats show slightly greater risk for the trait (+6%) than do males (Edwards, King, & Fray, 2000). This predicted increased risk in females was supported by our findings that the

greatest group difference in long-term extinction freezing behavior relative to same-sex controls was found in the female helpless subjects.

Our data also showed that males exhibited greater freezing than females. However, the larger size of the male rats may account for some of this sex difference. Being larger in size, males were housed one per cage whereas females were housed 2-3 per cage, resulting in more social isolation for the males. A larger computer-defined subject size, which is used to differentiate the subject's position from its movement, was also used for the males. The greater immobility in males may also be related to baseline sex differences in open-field behavior, since males show less motor activity than females in the open field (Archer, 1975; Furchtgott, Wechkin, & Dees, 1961; Seliger, 1977).

Conclusions

Congenitally helpless rats showed somewhat enhanced acquisition and dramatically impaired extinction of a traumatic memory—a crucial part of the behavioral phenotype associated with PTSD (Charney et al., 1993). The helpless rat has also shown differences in glucocorticoid regulation, conditioned analgesia, and regional brain metabolism which resemble biological abnormalities detected in PTSD patients. Thus, congenitally helpless rats may be a useful model for studying those biological factors which render a subset of individuals vulnerable to PTSD.

Chapter 4: Behavioral Effects of Metyrapone on Pavlovian Extinction

INTRODUCTION

Cordero, Kruyt, Merino and Sandy (2002) found that Pavlovian acquisition memory can be impaired by a dose of 50 mg/kg metyrapone, a corticosterone synthesis inhibitor, injected 90 minutes before training. We hypothesized that the same metyrapone treatment given before Pavlovian extinction may also impair extinction memory, and thereby facilitate recovery of the extinguished behavior. This hypothesis was tested in mice by examining the effects of 50 mg/kg metyrapone on the extinction of conditioned freezing following Pavlovian tone-shock conditioning. Metyrapone is a drug that inhibits glucocorticoid synthesis (corticosterone in rats and mice, cortisol in humans) by inhibiting 11-beta-hydroxylase, a rate-limiting enzyme in corticosteroid synthesis (Haynes Jr., 1990). Metyrapone in the rat stimulates the release of the corticosterone precursor deoxycorticosterone into the systemic circulation, where deoxycorticosterone can be converted into several neurosteroids (Strashimirov & Bohus, 1966). Metyrapone also increases plasma levels of adrenocorticotropin hormone (ACTH), because a decrease in corticosterone synthesis results in reduced negative feedback to the pituitary (Rotllant, Ons, Carrasco, & Armario, 2002).

In the case of extinction of conditioned behavior, plasma corticosterone levels in rats increased during extinction of a lever-press operant response (Coover, Goldman, & Levine, 1971) and metyrapone prevented the extinction of a conditioned emotional response in rats (Hernandez-Poudevida, McEwen, & Quirk, 2002). Administration of corticosterone or ACTH has led to the suggestion that increased corticosterone levels, rather than ACTH levels, improve the extinction of an operant response (Garrud, Gray, & de Wied, 1974; Hennessy, Cohen, & Rosen, 1973). Generally, extensive findings in the

literature suggest that corticosterone administration does not affect learning performance, but dose-dependently enhances memory consolidation (Cahill & McGaugh, 1998; Hui et al., 2004; Lupien et al., 1997; Pugh, Fleshner, & Rudy, 1997; Zorawski & Killcross, 2002).

We treated mice using specific parameters (50 mg/kg s.c. metyrapone, 24 hour retention test, 0.5 mAmp footshock) that effectively impaired Pavlovian acquisition memory in rats as reported by Cordero et al. (2002). A 90 minute interval after 50 mg/kg metyrapone effectively inhibits corticosteroid synthesis on the basis of the available behavioral and endocrinological studies (Cordero et al., 2002; Loscertales, Rose, & Sandi, 1997; Roozendaal, Carmi, & McGaugh, 1996). Our mice were trained on a Pavlovian extinction paradigm modeled after our recent brain metabolic mapping study (Barrett et al., 2003), and we investigated how this metyrapone treatment affects extinction performance as compared to extinction memory.

METHODS AND MATERIALS

Subjects

Subjects were 15 male CBA/J mice from Jackson Laboratory (Bar Harbor, ME), that weighed an average of 25 g at the start of training. Subjects were housed under standard laboratory conditions, 2-3 per cage, with a 12-hour light/dark cycle (0600-1800 hr lights on) and ad libitum food and water. Subjects were handled daily for 7 days prior to Day 1 of training. Training and testing was conducted between 1300 and 1600 hr. Animal experimentation was approved by the Institutional Animal Care and Use Committee and complied with all applicable federal and NIH guidelines.

Apparatus

The training apparatus for the acquisition phase (context A) consisted of a conditioning chamber (22 x 14 x 22 cm) (Med Associates, St. Albans, VT) enclosed in a sound-attenuated box illuminated by a red light. Two sides of the chamber were aluminum, with clear Plexiglas for the front, back and top. Tones were generated by two Waketek Sweep/Modulation generators (Wavetek, San Diego, CA) and presented through speakers mounted in the top of each chamber. The conditioned stimulus (CS) was a frequency-modulated tone of 1-2 kHz, two sweeps per second, 15 sec in duration, with an intensity of 65 dB, measured at the center of the floor of the chamber. The unconditioned stimulus (US) was an electric footshock of 0.5 mA, 0.75 sec delivered by a Lafayette Instruments Master Shocker (Lafayette Instrument Co., Lafayette, IN). Presentations of stimuli were controlled by computer programs, created using the MED-PC for Windows programming language (Med Associates). Between sessions the chambers were washed with a BioClean solution.

The extinction training occurred in a different context (context B): a clear plastic cage (19 x 25 x 15 cm), with a speaker mounted in the lid. Between sessions, each extinction box was cleaned with Betadine to provide a distinctive olfactory environment.

Behavioral training

Conditioned behavior

The conditioned response (CR) was freezing behavior, operationally defined as the mouse having all four feet on the floor, with minimal head movements and shallow, rapid breathing for at least 3 sec. The CS was conditioned to elicit a freezing response through temporally coincident pairing with the US. Each 15-sec tone CS was divided into five 3-sec bins, with the subject's behavior scored for each of the five bins.

Experimental design

The time course of training and probe trials is illustrated in Table 4.1. Both experimental groups underwent acquisition training (tone-shock pairings) for two days, followed by a day of extinction training (tone-alone presentations) under saline or metyrapone conditions. The groups were balanced according to their fear response during acquisition training. Subsequent tone-alone probe trials (without saline or metyrapone injections) measured the level of freezing in both training contexts.

Phase I

Days 1-2 consisted of habituation to context A. During this period, each subject explored the chamber for 1 hr, with no tones or shocks. Days 4-5 of training were again conducted in context A and consisted of acquisition training for both groups. Daily acquisition training consisted of four tone-shock presentations over 15 min, with intertrial intervals of 2, 2.5, 3, 3.5, or 4 min, randomly shuffled by the MED-PC program. During each trial of acquisition training, the 15-sec tone and 0.75-sec footshock were co-terminating.

Phase II

Day 6 consisted of extinction training in a new context (context B), to minimize the effects of excitatory conditioning to context A. During this 1 hr period, all subjects received 60 tone presentations of 15 sec each, with 45 sec between each tone CS, a schedule designed to produce robust extinction based on our previous study (Barrett et al., 2003). All subjects were injected subcutaneously, to the back of the neck, 90 min before the extinction session. Subjects in the saline group (n = 7) received a 0.1 mL injection of 0.9% sterile saline, while subjects in the metyrapone group (n = 8) received an injection of 1.25 mg of metyrapone [2-methyl-1,2-di(3-pyridyl)-1-propanone; Sigma-

Table 4.1: Experimental design of metyrapone experiment

	Context A		Context B			Context A
Group	**Day 1-2**	**Day 4-5**	**Day 6**		**Day 7**	**Day 9**
Metyrapone	Habituation No Tone, No Shock	Acquisition Tone –> Shock	Metyrapone Injection	Extinction Tone	Probe-E Tone	Probe-A Tone
Saline	Habituation No Tone, No Shock	Acquisition Tone –> Shock	Saline Injection	Extinction Tone	Probe-E Tone	Probe-A Tone

Probe-E, extinction context probe trials; Probe-A, acquisition context probe trials.

Aldritch] in 0.1 mL of 0.9% sterile saline. This dose of metyrapone corresponded to 50 mg/kg body weight, a dose previously shown to affect memory consolidation in rats when administered 90 min before training (Cordero et al., 2002).

Probe trials

Two sets of probe trials measured conditioned freezing in both groups and in both the acquisition and extinction training contexts. Behavior was recorded for the 15 sec prior to tone onset, as well as the subsequent 15 sec during tone CS presentation, to provide a comparison between freezing with and without the CS. No subject received either metyrapone or saline prior to probe trials. On Day 7, probe trials were conducted in context B (the extinction context). This consisted of 4 tone-alone presentations in 10 min, with 2 min between each tone, and freezing behavior scored as described above. On Day 9, another set of probe trials was conducted in context A (the acquisition context); the parameters were identical to the previous probe trial session. Nonparametric scores of 0-5 for freezing behavior during the 15-second pre-CS periods and the 15-sec tone CS periods were compared across groups using SPSS non-parametric Mann-Whitney U-tests (two-tailed, asymptotic significance $p < 0.05$).

Corticosterone assay

A final experiment was done to confirm that blood serum levels of corticosterone in mice were significantly reduced after metyrapone injection as compared to saline injection. Mice trained and treated with saline ($n = 7$) or metyrapone ($n = 7$) as described above were decapitated 90 minutes after injection, and trunk blood was collected and kept on ice. The samples were centrifuged, and supernatant was collected and stored at -80º C. Blood corticosterone concentrations were determined using an immunoassay kit

(Corticosterone Colorimetric Kit, Assay Designs, Inc., Ann Arbor, MI) with a sensitivity of 27 pg/mL. The intra-assay coefficient of variance was 11.9%. SPSS one-way ANOVA was used to test the significance (two-tailed, $p < 0.05$) of the differences between mean corticosterone levels in metyrapone and saline groups.

RESULTS

Behavioral results

The behavioral results indicated that the metyrapone treatment did not affect acquisition or extinction performance. The within-session extinction curves were not different between metyrapone and saline groups. However, metyrapone administered 90 min before extinction increased the recovery of the extinguished conditioned response to the tone in subsequent probe trials performed one and three days after extinction training. Table 4.2 summarizes the percentage freezing in each of the behavioral tests.

Post-acquisition probe

To verify that both groups acquired the tone-shock association, freezing behavior was probed on the day after acquisition with four tones in the extinction context. Conditioned freezing was equally manifested in both groups ($U = 14$, $p = 0.094$), as shown in Figure 4.1.

Extinction session

Conditioned freezing scores were averaged across the extinction session, and showed no significant group differences in freezing ($U = 27$, $p = 0.908$). The extinction curve is presented in Figure 4.2.

Table 4.2: Percent freezing to tone CS under metyrapone/saline conditions.

Test	Metyrapone mean ± s.e.	Saline mean ± s.e.
Post-acquisition probe	78 ± 2	84 ± 4
Extinction session	27 ± 2	28 ± 4
Extinction context probe	69 ± 9 *	42 ± 6
Acquisition context probe	96 ± 2 *	70 ± 6

* $p < 0.05$

Figure 4.1: Freezing counts during the first four tone CS presentations of the extinction session measured the levels of the CR after acquisition under metyrapone/saline conditions. Each 15-sec tone CS was scored in five 3-sec bins for freezing behavior.

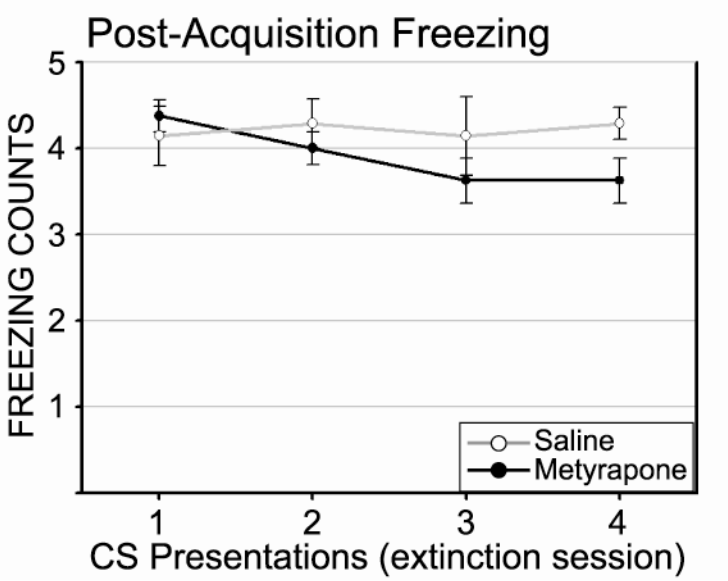
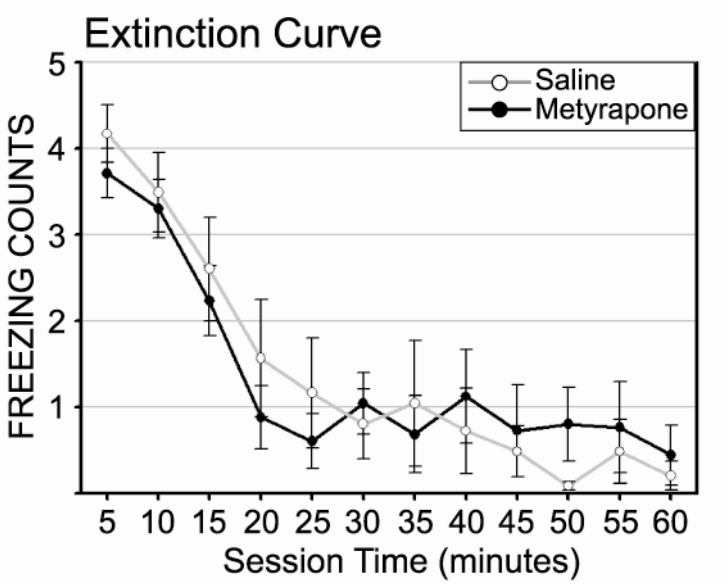


Figure 4.2: Freezing counts during 60 tone CS presentations in the 60-min extinction session under metyrapone/saline conditions measured the extinction curve. Each 15-sec tone CS was scored in five 3-sec bins for freezing behavior and averaged into 5-min bins.



Extinction context probe

The effects of metyrapone injection were seen one day after extinction, when subjects were probed with tone presentations in the extinction context (Figure 4.3). Metyrapone subjects froze more frequently than controls on probe trials CS1 ($U = 11.5$, $p = 0.037$) and CS3 ($U = 10.5$, $p = 0.04$). During the first tone CS presentation of the four probe trials, 5 out of 8 metyrapone subjects were maximally frozen (a score of 5/5), while only 1 out of 7 saline subjects was maximally frozen. Average freezing levels were 69% for the metyrapone group and 42% for the saline group. There was no group difference in pre-CS freezing scores averaged across the four trials ($U = 21.5$, $p = 0.447$).

Renewal test

When returned to the acquisition context and probed with tone presentations three days after extinction training, the group difference in freezing was more pronounced (Figure 4.4). The averaged probe trials showed significant differences in freezing across groups ($U = 6.5$, $p = 0.012$), with metyrapone subjects freezing more than controls in each trial (CS1: $U = 8$, $p = 0.005$; CS2: $U = 10$, $p = 0.018$; CS3: $U = 4$, $p = 0.002$; CS4: $U = 7$, $p = 0.012$). During the first three CS presentations of the four probe trials, 7 out of 8 metyrapone subjects remained maximally frozen, while only 1 out of 7 saline subjects was maximally frozen. Average freezing levels were 96% for the metyrapone group and 70% for the saline group. There was no group difference in pre-CS freezing scores averaged across the four trials ($U = 27.5$, $p = 0.922$).

Corticosterone assay

The results confirmed that blood serum levels of corticosterone in trained mice were significantly lower ($F_{(1,13)} = 5.5$, $p = 0.036$) 90 min after metyrapone injection (mean ± SEM = 14.1 ng/mL ± 2.6) than 90 min after saline injection (49.0 ng/mL ± 14.6).

Figure 4.3: Freezing counts during four tone CS presentations in post-extinction probe trials in the extinction context measured retention of the CR. Each 15-sec tone CS was scored in five 3-sec bins for freezing behavior. Pre-CS freezing was scored for the 15 sec before tone onset and averaged across the four trials. *Mann-Whitney U-test significant at $p < 0.05$.

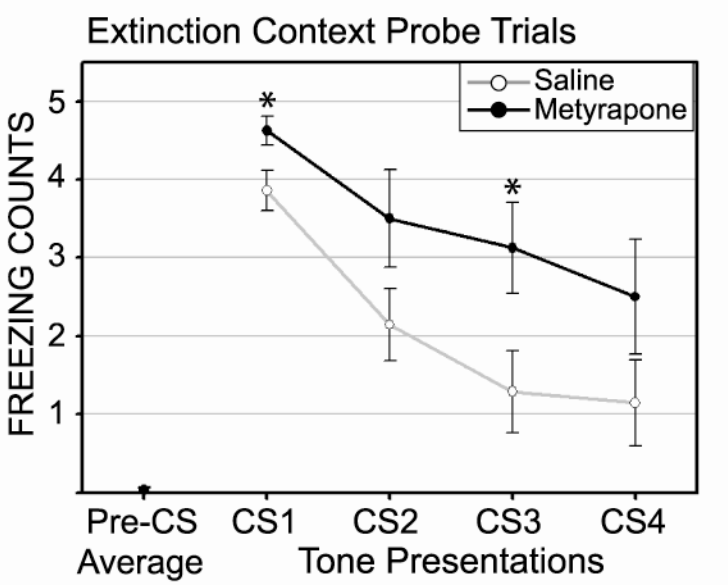
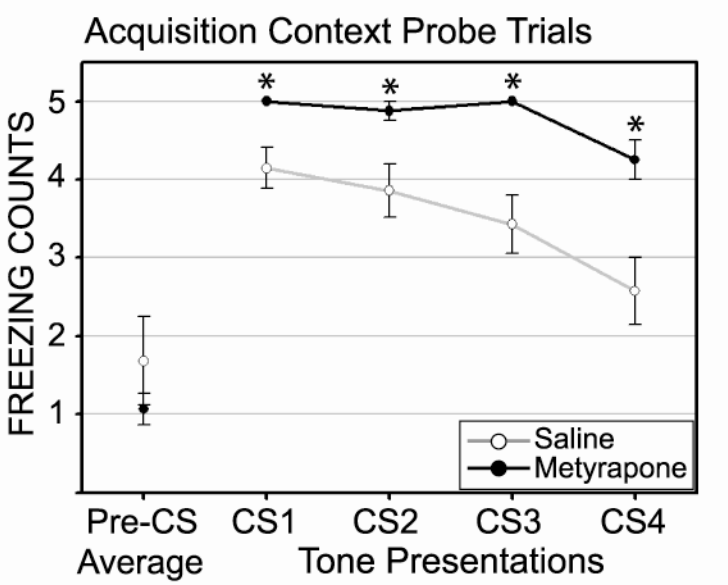


Figure 4.4: Freezing counts during four tone CS presentations in post-extinction probe trials in the acquisition context measured retention of the CR. Each 15-sec tone CS was scored in five 3-sec bins for freezing behavior. Pre-CS freezing was scored for the 15 sec before tone onset and averaged across the four trials. *Mann-Whitney U-test significant at $p < 0.05$.



DISCUSSION

The lack of group differences in acquisition and extinction performance, together with the similar pre-CS freezing behavior of saline-treated and metyrapone-treated mice, suggest that nonspecific motor or performance deficits were not produced by our metyrapone treatment. The post-acquisition freezing data (Figure 4.1) indicated that both groups showed similar initial levels of freezing, suggesting that the formation of a tone-shock association after acquisition training was not affected by the metyrapone treatment. The extinction results (Figure 4.2) showed that metyrapone did not affect the extinction curve for a tone-conditioned Pavlovian freezing response.

Interestingly, metyrapone treatment during extinction modified the subsequent recovery of conditioned freezing evoked by the tone. Mice treated with metyrapone showed a greater amount of tone-evoked conditioned freezing in the probe sessions conducted one and three days after extinction training. This enhancing effect of metyrapone was found in both the extinction (Figure 4.3) and acquisition (Figure 4.4) contexts. The acquisition context allowed conditioned freezing to be expressed maximally in metyrapone subjects for three tone CS presentations. However, there were no group differences in pre-CS freezing behavior, suggesting that the effects of metyrapone were specific to the CS-evoked freezing.

The two probe trials conducted one and three days after metyrapone injection occurred in the extinction and acquisition contexts, respectively. By definition the restoration of the extinguished behavior in these two contexts represents the difference between the behavioral phenomena called spontaneous recovery by Pavlov (1927) (extinguished CR reappears over time in the extinction context) and renewal by Bouton (2002) (extinguished CR reappears with a return to the acquisition context). A greater

restoration in CR expression was clearly present in the acquisition context. Since metyrapone subjects never received metyrapone or tone-alone CS presentations in the acquisition context, a state-dependent effect of metyrapone on the renewal of the CR can be ruled out. While these two forms of restoration of the CR may rely on different context-dependent mechanisms, only impairment of the CS-specific extinction memory by metyrapone could explain the enhanced restoration of the CR in both cases. Metyrapone action, during the extinction phase with the CS in context B, may be expected to selectively impair retention of the CS-specific extinction memory while not affecting the contextual memory to context A formed during acquisition.

Glucocorticoids have been implicated in operant extinction (Garrud et al., 1974; Hennessy et al., 1973) and contextual Pavlovian extinction (Smotherman & Levine, 1978). Cordero et al. (2002) found that metyrapone injection prevented increased blood corticosterone concentration after contextual fear conditioning, and also dose-dependently impaired the retention of the contextual freezing CR, 24 hours later, in rats conditioned with either 0.4 or 1 mAmp footshocks. The parameters of our experiment (50 mg/kg s.c. metyrapone, 24 hour retention test, 0.5 mAmp footshock) model these, but the metyrapone injection occurred during extinction, not acquisition. When metyrapone was administered before acquisition, Cordero et al. (2002) found less freezing when tested later, but when metyrapone was administered before extinction, our study found more freezing in probe trials. Cordero et al. (2002) used the acquisition context alone for probe trials, and still saw a difference in freezing behavior. We used a tone CS during probe trials to measure evoked freezing behavior between contexts, and found a larger difference. Therefore, the behavioral expression of the CR is different, but in both studies the effects of metyrapone may be explained by impairment in associative memory retention. That is, metyrapone may impair the retention of the CS-US memory formed

during acquisition, or the CS-no US memory formed during extinction, depending on whether metyrapone is administered before acquisition or before extinction, respectively.

Adrenalectomy can impair the extinction of passive avoidance in rats, and that effect can be normalized with corticosterone (Bohus & De Kloet, 1981). However, in that study adrenalectomy was done one hour before extinction training, which may impair the initial performance of the rats. No initial measure of how adrenalectomy affected extinction performance was presented. Behavior was only evaluated 24 hrs later. The design of the Bohus and de Kloet (1981) study cannot differentiate between performance and consolidation effects. In contrast, our study with metyrapone showed that the performance of mice during extinction was not affected. It was the retention of extinction tested one and three days later that was affected.

An impairment of the consolidation of the extinction memory could explain our findings in which the extinction session behavior itself was unimpaired after metyrapone injection, but the recovery and renewal of conditioned freezing after extinction training was enhanced. Our results are also consistent with previous studies that suggest that inhibition of corticosteroid function interferes with memory consolidation for a variety of learned responses to footshock in rodents. For example, corticosterone effects on memory consolidation of tone-shock Pavlovian conditioning have been reported (Hui et al., 2004; Pugh et al., 1997; Zorawski et al., 2002). Similar effects have been found in inhibitory avoidance and contextual fear conditioning (Cordero et al., 2002; Roozendaal, Bohus, & McGaugh, 1996). Amnesic effects following inhibition of corticosteroid function have also been found in many other learning tasks and species (Lupien et al., 1997; Oitzl & De Kloet, 1992). The facilitating effects of stress on memory consolidation are blocked by metyrapone (Liu, Tsuji, Takeda, Takada, & Matsumiya,

1999; Roozendaal et al., 1996), suggesting that endogenously released stress hormones play a role in memory consolidation (Hui et al., 2004).

Corticosterone binds to two receptor types: mineralocorticoid (Type 1) and glucocorticoid (Type 2). Because Type 1 receptors have a higher affinity for corticosterone than Type 2 receptors, any reduction in corticosterone levels would affect the glucocorticoid receptors more than the mineralocorticoid receptors. Extensive evidence indicates that the memory effects of corticosteroids are mediated selectively by binding to the low affinity glucocorticoid Type 2 receptor (Lupien et al., 2002; McEwen, De Kloet, & Rostene, 1986). This evidence suggests that metyrapone treatment may have affected extinction memory by reduction of corticosterone binding mainly to glucocorticoid receptors.

An alternative explanation for the observed behavioral results may not involve glucocorticoid inhibition, because metyrapone not only suppresses corticosterone synthesis, but it also has other actions. For example, metyrapone dose-dependently increased plasma levels of ACTH (Rotllant et al., 2002), due to reduced negative feedback regulation. The observed behavioral effects of metyrapone may be related to ACTH increase. The effects of direct administration of ACTH have not been assessed in Pavlovian extinction, but Hennessy et al. (1973) showed that increased corticosterone levels, rather than ACTH levels, affected the extinction of an appetitive runway response. Therefore, while it is impossible to rule out ACTH effects on our observed results without further experiments, the available studies suggest a more important role of corticosterone for memory consolidation in extinction. Metyrapone also stimulates the systemic release of deoxycorticosterone (Strashimirov et al., 1966), the corticosterone precursor, which can be converted into several neurosteroids, such as tetrahydroxydeoxycorticosterone, which may contribute to the observed behavioral

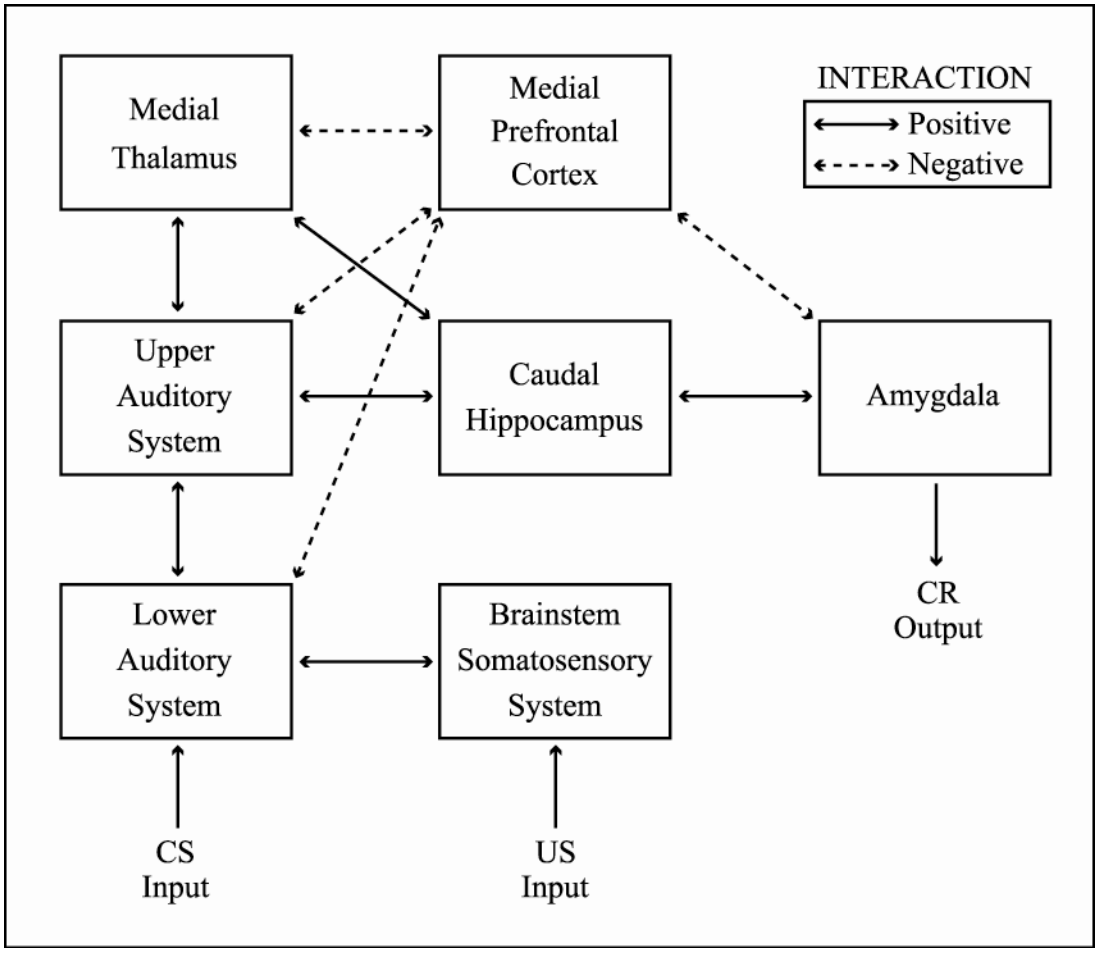
effects. Therefore, the possibility of metyrapone acting through various mechanisms besides corticosterone inhibition cannot be ruled out. Metyrapone may also affect brain regions linked to extinction (Barrett et al., 2003; Milad et al., 2002; Nair et al., 1999), which may contribute to the observed amnesic effects. Further studies are needed to identify regions affected by metyrapone action during Pavlovian extinction.

Chapter 5: A Functional Network Model of Pavlovian Extinction

On the basis of the brain metabolic and behavioral results discussed here, we propose that extinction training forms a new functional network of interactive brain regions, which serves two crucial functions: to inhibit the CR after extinction, and to preserve the original CS-US association from acquisition. Pavlov's (1927) original inhibitory hypothesis is supported by our results. The competing hypothesis, of extinction as a form of unlearning, is less likely, because even regions showing significant excitatory effects, which returned to their pre-acquisition activity after extinction, showed more significant inter-regional interactions after extinction than after acquisition alone. The post-extinction decreases in activity may actually reflect CR inhibition rather than unlearning.

The neural network formed after Pavlovian extinction is functionally divided according to the specializations of the regions and systems involved. We propose that the framework of Pavlovian conditioning paradigms, in terms of the CS, US, and CR, can be used to understand the brain metabolic effects seen here. The elements of both acquisition and extinction learning are represented in this model, including the CS (auditory system), the US (brainstem somatosensory system), contextual modulation of CS-US contiguity (hippocampus), the expression of the CR (amygdala), and the inhibition of the CR (prefrontal cortex), while the medial thalamus provides a relay between prefrontal cortex and its subcortical targets of inhibition. A diagram of these interactions, generalized from our pairwise inter-regional correlation findings, is presented in Figure 5.1.

Figure 5.1: Proposed functional network underlying Pavlovian extinction.



CS PATHWAYS

The consistent findings in FDG conditioning studies using tone CSs are tone-evoked activity changes in auditory system regions. Enhancement of activity in CS-specific sensory systems is commonly observed in our lab's deoxyglucose conditioning studies (Gonzalez-Lima, 1992), indicating that the activity of these auditory regions reflects the associative value or salience of a particular CS. CS pathways are clearly modified when the CS acquires a meaning, beyond its properties as a tone. But regions within this system may be functionally specialized for different kinds of activity changes and interactions after acquisition vs. extinction. The greater activity evoked by the CS in the dorsal cochlear nucleus after acquisition was also observed after extinction, suggesting that the CS signal value is not "lost" or "unlearned" after extinction. But the relatively decreased activity in some higher auditory system regions (TE3) found in our extinction group could be the result of an "unlearning" process similar to habituation.

Tone-evoked changes in the auditory system were observed in our lab's FDG study of Pavlovian differential inhibition (Jones et al., 2001a). In this paradigm, a tone inhibitor (CS-) is presented alone, and a light excitor (CS+) cues a footshock US. The control group is trained on tones, lights and shocks presented pseudorandomly. The FDG test with the CS- tone inhibitor produced activity decreases in auditory relay nuclei (anterior ventral cochlear nucleus and central inferior colliculus), which showed significant acquisition-related increases but not extinction effects in Barrett et al. (2003). The effects of a tone CS- in the auditory system were opposite to those found in our non-extinction group, likely the result of excitatory conditioning to a tone CS+, i.e., activity increases in VCA and ICC.

More tone-evoked effects in CS pathways were found in our lab's FDG study (Jones et al., 2001b) of the blocking effect (Kamin, 1969). The Kamin blocking effect is an example of how subjects use their current knowledge to learn new responses (Domjan, Cusato, & Villarreal, 2000). In this paradigm, the presentation of a previously conditioned excitatory stimulus (CS1) will retard or "block" the CR to a novel stimulus (CS2) when they are presented in compound. Jones and Gonzalez-Lima (2001b) tested the blocking effect against tone excitor and pseudorandom groups, and found tone-evoked effects in several systems. Barrett et al. (2003) replicated the findings of Jones and Gonzalez-Lima (2001b) in auditory cortex (TE1, TE3) and inferior colliculus (ICE, ICC), in which a tone CS+ resulted in increased FDG uptake. These increases were found in both the tone excitor and tone blocked groups, and also found in our non-extinction group, but not in our extinction group. These findings may be elucidated by an analysis of how the CS-US contingency differs between these four groups.

In the tone-excitor, tone-blocked, and non-extinction groups, every tone CS presentation was paired with a footshock US. Only our extinction group received tone-alone presentations after excitatory conditioning. Both the tone-excitor, tone-blocked, and non-extinction groups showed an activational effect in these auditory system regions, regardless of the expression of the freezing CR (excitor = CR, blocked = no CR; non-extinction = CR). The persistent activity of structures like auditory cortex and inferior colliculus may represent the CS-US contingency which is common to the tone excitor, tone blocked, and non-extinction groups. When this contingency is weakened, as in extinction training, these auditory regions show a decrease in activity. The involvement of inferior colliculus in CS-US representations is also supported by our extinction group's extensive functional coupling between the lower auditory system and the external cuneate, which is discussed later as a possible US pathway.

The upper auditory system showed a more complex pattern of effects in our extinction group. Medial geniculate and auditory cortex were inversely correlated with prefrontal cortex, and uncoupled from the lower brainstem network of auditory regions and external cuneate. Also, of the higher regions, only the ventral medial geniculate showed significant increases after acquisition which persisted after extinction. Auditory cortex TE3 showed the opposite effect: a significant increase after acquisition which was significantly reversed after extinction. Thus the upper auditory regions are represented as a separate system in Figure 5.1, one which is more interactive with frontal cortical, hippocampal and thalamic regions. However, the significant negative correlation between frontal cortex and dorsal cochlear nucleus indicates that prefrontal inhibition may also act on the lower auditory structures.

US PATHWAYS

The external cuneate nucleus (ECu) in the brainstem, which may modulate the value of the footshock US during Pavlovian conditioning, was functionally coupled to a network of lower auditory regions (lateral lemniscal nuclei, inferior colliculus, and ventral cochlear nucleus) after Pavlovian extinction, but not after acquisition (Barrett et al., 2003). The ECu projects to the thalamus (Mantle-St John & Tracey, 1987), to the cerebellum (Berretta, Perciavalle, & Poppele, 1991) and to brainstem regions involved in cardiovascular, respiratory and sensorimotor control (Lan et al., 1994), and has been implicated in somato-autonomic reflex responses (Dean & Kostreva, 1987). The ECu represents the first step of potential interaction between CS and US pathways during acquisition, which could account for both the excitatory effect seen in the non-extinction group and the significant correlations with lower auditory regions in the extinction group.

The external cuneate was sampled by Jones and Gonzalez-Lima (2001b) in our lab's FDG study of blocking, at the corresponding level as in Barrett et al. (2003). The ECu showed significantly increased activity in the tone-blocked relative to the tone-excitor group (Jones et al., 2001b), as well as significantly increased activity after acquisition but not extinction (Barrett et al., 2003). In each of these groups, the metabolic activity was tone-evoked; there were no footshock US presentations for any subject during FDG uptake. Also, for each of these group comparisons in both blocking and extinction, all subjects have experienced the same number of footshock US presentations during training. Therefore, any group differences in US regions like the ECu must be linked to the subject's prior experiences with both the CS and the footshock US.

Why does the external cuneate show significantly increased activity in our non-extinction group relative to pseudorandom (Barrett et al., 2003), but increased activity in the blocking group relative to the tone-excitor group (Jones et al., 2001b)? Both the non-extinction group and tone CS+ excitor group underwent acquisition training of tones paired with footshock. Again, the differences in behavioral training provide a framework for understanding the effects. Blocking entails pre-exposure not just to the initial excitatory CS but to the US as well. In our Pavlovian extinction design, there were no US pre-exposures. Also, our non-extinction group was not tested immediately after excitatory acquisition training; otherwise, this group would be identical to the tone-excitor group. Instead, this group was exposed to the extinction context, for two hours over two days, in the absence of tones, to match the time spent in this context to the extinction group undergoing two days of tone-alone presentations. Thus, context habituation after acquisition is another factor different between the tone excitor group and the non-extinction group.

These effects of US pre-exposure and context habituation may account for the activity changes seen in the external cuneate. The blocked tone CS2 is the most novel stimulus to be paired with the footshock US, since both the excitatory CS1 and US have been previously presented. In the non-extinction group, the original CS-US association is intact, and the original tone CS has retained its meaning. But the spinocerebellar pathways and the external cuneate may become more activated by the prior exposure to the footshock US in the blocking group, especially when the US is presented again in compound with a tone CS. Also, the modulation of the ECu may be context-dependent; our lab's recent FDG study of the renewal effect showed significant increases in the external cuneate in the renewal group, which was exposed to the acquisition context during the FDG session (Bruchey and Gonzalez-Lima, in preparation). Thus, the tone-evoked FDG increases in the ECu could be similar in the blocked and non-extinction group, if the US representation in the external cuneate is modulated by experiences with the US, the context, and not the most *reliable* CS+, but rather the most *recent* CS+.

The external cuneate may relay spinocerebellar information not just to the cerebellum, which showed both excitatory and contiguity effects in the Jones and Gonzalez-Lima (2001b) blocking study, but also to the lower auditory system, which showed increased functional coupling with the external cuneate after extinction but not after acquisition (Barrett et al., 2003). The ECu may function as a crucial node in the most basic circuit mediating the original tone-footshock memory, at the level where tone CS and footshock US information meet in the brainstem. The possible context dependence of ECu's activity underscores the fact that even brainstem regions might be influenced by an upstream functional network of cortical and midbrain regions.

CS-US CONTIGUITY AND CONTEXT

The caudal (ventral) hippocampus showed effects in both the Jones and Gonzalez-Lima (2001a) study on differential inhibition, the Shumake et al. (2002) study on congenitally helpless rats, and in our extinction study (Barrett et al., 2003) at the same caudal level. Of the four regions showing significant contiguity effects in Barrett et al. (2003), three (presubiculum, subiculum and cCA3) are involved in hippocampal processing. These regions showed significantly increased activity after acquisition which persisted after extinction. The extinction group received more CS presentations and did not show a CR; thus the effects in caudal hippocampus common to both extinction and non-extinction groups are likely due to the original CS-US association.

While the Jones and Gonzalez-Lima (2001a) FDG study of differential inhibition found a decrease in activity in the caudal hippocampus with a CS-, we found significant activity increases from both a CS+ and an extinguished CS in presubiculum, subiculum and cCA3. In other words, the significant activity differences in these regions were reversed between a CS+ and a CS-, but did not change between a CS+ and an extinguished CS+. Understanding the difference between a tone inhibitor and an extinguished tone may provide support for the argument that the hippocampus is involved in CS-US contiguity detection, and may represent elements of the remaining memory of acquisition after extinction.

The crucial difference between a tone inhibitor and an extinguished tone is that the CS- has never been paired with a footshock US. The extinguished tone was excitatory, then inhibitory; the CS- is a safety signal. The extinguished CS has inverted its meaning; the CS- is inhibitory, and always has been. When viewed in terms of the previous CS-US contingency, the two stimuli, while both inhibitory in terms of behavior, are fundamentally different. In the case of the differential inhibitor, there is no previous

excitatory association to be modified on the basis of tone-evoked stimuli. There is no CR or CS-US contingency to be inhibited. And as such, no effect was found in prefrontal cortical regions by Jones and Gonzalez-Lima (2001a); in fact, the trend was for decreased activity in mPFC from the tone inhibitor, perhaps reflecting the behavioral disinhibition provided by the CS-. However, the similar activational effects in the hippocampus between a CS+ and extinguished CS reflect the original CS-US contingency in the training paradigms. Even after extinction, the increase in metabolic activity persists, perhaps representing the original CS-US contingency, regardless of the CR.

The caudal hippocampus was also implicated in our lab's FDG study of extinction of instrumental conditioning in rat pups (Nair et al., 1999). In this paradigm, rat pups of two ages [12-day-old (P12) and 17-day-old (P17)] are trained to acquire an instrumental response (running down a runway) to achieve an appetitive reward (milk from an anesthetized dam), then trained on extinction. The P17 pups showed faster extinction rates, and elevated activity in subiculum, CA1 and CA3 hippocampus, relative to P12 pups. A functional network between septal, hippocampal and ventral tegmental regions was found in the P17 pups, but only after extinction training, not in handled controls that lacked the original conditioning (Nair et al., 1999).

A large body of literature has documented the involvement of the hippocampus in Pavlovian conditioning. Extinction can be facilitated (Richardson et al., 2004) or retarded (Corcoran et al., 2001) with NMDA agonists and antagonists in the hippocampus, respectively. Caudal regions of the hippocampus are specialized for dealing with fear and anxiety (McHugh, Deacon, Rawlins, & Bannerman, 2004). Lesions of caudal (but not rostral) hippocampus or subiculum reduce levels of freezing after footshock (Maren, 1999; Maren, Aharonov, & Fanselow, 1997; Richmond et al., 1999;

Bannerman et al., 2003) and reduce unconditioned anxiety as well (Bannerman et al., 2002; Bannerman et al., 2003; Kjelstrup et al., 2002).

Other parts of the hippocampus may be specialized to deal with the contextual modulation of the memory for fear extinction (Corcoran et al., 2004). Microinjection of amphetamine into dorsal hippocampus increased conditioned freezing to the excitatory context but not to the tone CS (White & Salinas, 2003). The effects we see in caudal hippocampus may be due to the tone CS used during the FDG session, and the fact that the subjects were tested in the extinction context, not the excitatory acquisition context.

The congenitally helpless rat shows baseline hypermetabolism in the caudal (but not rostral) hippocampus and subiculum (Shumake et al., 2002). If these regions are indeed involved in the modulation of the CS-US association, the abnormally high baseline activity in the congenitally helpless rat may predispose this strain to develop stronger excitatory CS-US associations, retarding the extinction process. This is consistent with the extinction deficit found in the congenitally helpless rat (Chapter 3).

However, the hormonal context may also interact with these hippocampal regions. The congenitally helpless rat shows increased metabolism in the paraventricular hypothalamus (Shumake et al., 2001), which is linked to stress hormone regulation via the hypothalamic-pituitary-adrenal (HPA) axis. In congenitally helpless rats, exposure to cues previously associated with stress resulted in impaired performance in a spatial memory task, as well as a blunted post-stress corticosterone response (King et al., 2001). In our experiment on wild-type mice, stress hormone intervention with metyrapone resulted in extinction retention deficits, as well as decreases in blood corticosterone levels (Barrett et al., 2004).

The caudal hippocampus is involved with feedback regulation of the HPA axis (Jacobson & Sapolsky, 1991), and lesions of caudal (but not rostral) hippocampus reduce

blood corticosterone levels after exposure to a stressful context (Kjelstrup et al., 2002). This could explain why the HPA axis disruption seen in metyrapone-treated mice and in congenitally helpless rats leads to an extinction deficit. These hippocampal regions may provide a crucial nexus for the interaction between the CS-US association, physical context, and hormonal context. This interactive property is reflected in the significant pairwise correlations between caudal hippocampus, thalamus and auditory system in Figure 2.10 and in the schematic Figure 5.1.

CR EXPRESSION

The excitatory effect in the central nucleus of the amygdala consisted of a significant increase in the non-extinction group relative to the extinction group, with the pseudorandom group midway between them. It could be due to excitation, inhibition or both processes. The change in the central nucleus is consistent with levels of the CR between groups (non-extinction = high freezing, pseudorandom = low freezing, extinction = lowest freezing). This nucleus is considered the output or response expression area of the amygdala, and its involvement with fear conditioning and the extinction of the freezing CR has been extensively documented (for review, see Pare, Quirk, & Ledoux, 2004).

In the Quirk/LeDoux model, acquisition occurs in the amygdala, and extinction involves prefrontal inhibition of the amygdala. This model cites the central nucleus of the amygdala as a "critical site of plasticity" (Pare et al., 2004) and infralimbic cortex as the inhibitory influence. However, other pathways in the auditory system, hippocampus and thalamus are also modified by both acquisition and extinction, regardless of the CR. The central amygdala may represent a final common output pathway for the freezing CR, which could account for the large body of literature regarding the central amygdala and

fear conditioning. But the Quirk/LeDoux model is too simplistic, as it neglects the interactions with other regions. The central amygdala shows increased functional coupling with anterior cingulate and nucleus accumbens in the congenitally helpless pup (Shumake et al., 2004). This increase in functional connectivity between limbic regions may also predispose this strain to the formation of stronger traumatic memories.

Medial prefrontal regions project to the amygdala (Freedman, Insel, & Smith, 2000; Mcdonald, Mascagni, & Guo, 1996; Sesack, Deutch, Roth, & Bunney, 1989), supporting the Quirk/LeDoux model. But the caudal hippocampus projects strongly to the amygdala as well (Henke, 1990; Krettek & Price, 1977; Petrovich, Canteras, & Swanson, 2001; Swanson & Cowan, 1977; van Groen & Wyss, 1990). For this reason, although no pairwise correlations with central amygdala remained significant after the jackknife procedure, pathways between the amygdala and prefrontal cortex, as well as between the amygdala and caudal hippocampus, were included in our model in Figure 5.1.

CR INHIBITION

The extensive literature on the critical inhibitory involvement of medial prefrontal regions in Pavlovian extinction supports both the Quirk/LeDoux model and our own. Extinction is impaired after lesions of infralimbic cortex (Morgan et al., 1993; Morgan, Schulkin, & LeDoux, 2003; Quirk, Russo, Barron, & Lebron, 2000), and infralimbic stimulation reduces the level of a freezing CR (Milad et al., 2002). The infralimbic cortex showed the single largest increase of the regions showing extinction effects in our study (+23%). However, medial frontal cortex showed the highest brain/behavior correlation with our extinction index (0.99), and dorsal frontal cortex showed significant negative inter-regional correlations, which were also present in mPFC and IL, but did not

survive the conservative jackknife procedure in any other frontal regions. Multiple frontal cortical areas, including medial, dorsal and infralimbic regions, play a role in extinction learning, and for this reason they are represented in Figure 5.1 as the prefrontal cortical system.

FDG studies of the partial reinforcement extinction effect in infant rats by Nair, Berndt, Barrett and Gonzalez-Lima (2001b; 2001a) revealed extinction effects in frontal cortex, centromedial and medial dorsal thalamus, and cerebellum (2001b). The older pups extinguished faster than the younger pups, and also showed extensive functional coupling between medial prefrontal, orbitofrontal, and anterior cingulate cortices. This network was not observed in the younger pups (2001a). Similar patterns of activity between extinction of operant and Pavlovian conditioning were found in medial frontal and medial thalamic regions, and functional networks were created as a result of both paradigms.

The formation of interactive networks, as manifested by significant inter-regional correlations, is facilitated after extinction of both Pavlovian and operant conditioning. Extinction learning, both Pavlovian and operant, is built on top of a previous association: the memory of acquisition. This may not overwrite the original association, but it may force the elements of the network to become more cooperative and functionally interconnected. Frontal cortical inhibition could be the factor that forces subcortical regions to become more correlated in activity. However, the increase in pairwise regional correlations seen after extinction could also be a property of the maintenance or re-consolidation of the memory for acquisition. During extinction training, the subject is repeatedly presented with the cue from the previous association, causing the reactivation of CS pathways and other regions linked to the original memory. In other words, the

increase in network properties after extinction training may result from either top-down (CR inhibition), bottom-up (CS-evoked activity), or a combination of both processes.

Blocking effects in medial prefrontal cortex can be explained through an analysis of how a blocked tone CS is different from an extinguished tone CS. Jones and Gonzalez-Lima (2001b) found significantly decreased mPFC activity in the tone blocking vs. excitor groups; we found significantly increased mPFC activity in the tone extinction vs. non-extinction groups. The crucial difference here is in CR inhibition. In the blocking group, a freezing CR never develops to the tone; in the extinction group, a freezing CR is first evoked by the tone, then inhibited. The blocked tone CS is not the same as a safety signal, as in differential inhibition, since the blocked CS has been paired with a footshock US. After extinction, the tone CS is a cue for CR inhibition; after blocking, the tone CS is a potential new cue for the footshock US. Behaviorally, the blocked CS appears to be inhibitory in that it reduces the expression of the tone-evoked freezing CR, when compared to subjects for whom both CSs are equally predictive of the US. However, the lack of CR found in the blocking effect is likely the result of arousal/attentional mechanisms activated by the previously conditioned CS+, and not the result of frontal cortical inhibitory mechanisms. In fact, the decrease in mPFC activity observed in the blocked group could indicate the potential disinhibition of CR expression, which may be adaptive for conditioning to a previously blocked CS which may yet hold predictive value.

The rostral caudate-putamen (rCPU) is also implicated in prefrontal cortical inhibition. The rCPU and medial parietal sensorimotor cortex showed elevated activity in our extinction group; these regions may serve as part of the sensorimotor US representation. The rCPU was also the most highly interactive region in the pseudorandom group, with connections between medial parietal cortex and medial,

dorsal, and lateral frontal cortical regions. These correlation coefficients with frontal regions were positive, not negative as in the extinction group. Activity changes in rCPU were also found in the Jones and Gonzalez-Lima (2001b) blocking study, at the corresponding level as in Barrett et al. (2003). The rCPU showed a contiguity effect, i.e., activity increases in both tone-blocked and tone-excitor groups relative to pseudorandom. The tone-blocked and tone-excitor groups shared the CS-US contiguity but not the CR.

Anatomical studies of connections with caudate-putamen have suggested functional differences between medial and lateral rCPU. The medial rCPU connects directly with auditory and medial prefrontal cortex (Heimer, Zahm, & Alheid, 1995; McGeorge & Faull, 1989), and connects directly or indirectly with the hippocampal formation (including subiculum, entorhinal cortex, and hippocampus proper) (Amaral & Witter, 1995). The lateral rCPU connects with sensorimotor cortex (McGeorge et al., 1989) and substantia nigra (Heimer et al., 1995). Functionally, the medial rCPU is more involved with limbic regions, while lateral rCPU is more involved with motor functions. On this basis, the FDG data from rCPU was separated into lateral and medial divisions, and examined separately with ANCOVA, as in the analysis presented in Appendix Table 6.1. The results showed that medial rCPU maintained significance at $p < 0.01$, but lateral rCPU did not meet the $p < 0.01$ level. Given the anatomical connections with other regions showing extinction effects (mPFC) and common effects (hippocampus), it is likely that more medial (limbic) subdivisions of rCPU are driving the extinction effect found there. The means, standard errors, and 99% confidence intervals for these subdivisions of rCPU are listed in at the end of Appendix Table 6.1.

Rostral CPU also showed increased functional coupling with infralimbic cortex in our non-extinction group. Infralimbic cortex is the region implicated in real-time extinction behavior by Milad and Quirk (2002). During the FDG session, the tone CS is

presented almost continuously, in the absence of footshock, to generate tone-evoked brain metabolic changes. These CS presentations (5-sec-on, 1-sec-off) are similar to the discrete CSs used in extinction training, which may evoke on-line, real-time, extinction-related changes in the brain, in our group which had never received excitatory tone CS presentations before. The rCPU-IL correlation in the non-extinction group may represent the brain's first steps towards inhibition of the CS-US representation. But after extinction training, this positive rCPU-IL correlation is gone, and rCPU activity is increased.

Medial prefrontal cortex may also account for the effects seen in metyrapone-treated and congenitally helpless subjects. Infusion of hydrocortisone facilitates PFC-dependent memory and PFC activation (Ganguli et al., 1994), and corticosterone appears to be required for normal function of mPFC (Mizoguchi, Ishige, Takeda, Aburada, & Tabira, 2004). Disruptions of both mPFC function and stress hormone regulation have been implicated in PTSD (for review, see Vermetten & Bremner, 2002). In infant congenitally helpless rats, brainstem regions are uncoupled from networks of frontal cortical and limbic regions (Shumake et al., 2004). This may indicate a developmental disorder in which brainstem regions are disconnected from the functional network formed after Pavlovian extinction, removing these lower regions from inhibitory influences.

The extensive literature on mPFC and Pavlovian extinction, and the results of our lab's previous FDG studies of extinction of instrumental conditioning (Nair et al., 2001a), differential inhibition (Jones et al., 2001a), and blocking (Jones et al., 2001b), as well as the brain/behavior correlations and negative functional coupling with other regions seen in Barrett et al (2003), provide compelling evidence for Pavlov's (1927) inhibitory mechanism of extinction. We conclude that frontal cortical regions serve to inhibit the CR after extinction by negatively interacting with amygdala, auditory and medial thalamic structures, as shown in Figure 5.1.

CONCLUSIONS

The mechanism for Pavlovian extinction is not found in any one brain system, even though frontal regions may be critical for successful extinction training. The changes in brain metabolic activity after extinction are widespread, in systems involving the CS (auditory system), the US (external cuneate), the contextual modulation of CS-US contiguity (hippocampus), the expression of the CR (amygdala), and the inhibition of the CR (frontal cortex).

Conditioning theory and brain metabolism are equally important to our interpretation of these findings. The complexity of Pavlovian extinction mechanisms is the result of new learning, built on top of the old. Extinction entails not just new learning, but the adaptive modification of old memories as well. In the process of modifying and updating the original memory for fear acquisition, structures in CS and US pathways become more interactive with thalamic, auditory and hippocampal regions. This process may serve to consolidate the excitatory CS-US association, even as the resulting CR is inhibited by frontal cortex. As a result, even after extinction, the original acquisition memory is still available; in fact, it is now subject to modulation by the same factors represented by this functional network: CS presentations (spontaneous recovery), US presentations (reinstatement), and context (renewal).

This is the first time such a functional network has been demonstrated after Pavlovian extinction. FDG autoradiography has the unique potential to non-invasively metabolically map any regional change and any neural network formed as the result of a learning process. This method provided a glimpse of the activity and interactivity of an intact brain, a snapshot of tone-evoked metabolic effects in a brain that has learned and subsequently modified the meaning of that tone after Pavlovian extinction.

Appendix Tables

Appendix Table 6.1: Results of covariance analysis (ANCOVA) of mean FDG uptake in extra-auditory regions.

Region	Bregma	Group	Mean	S.E.	Lower 99%	Upper 99%
Prelimbic frontal cortex (PrL)	2.22	Extinction	416	±23	348	484
		Non-extinction	365	±21	304	426
		Pseudorandom	355	±19	297	412
Medial prefrontal cortex (mPFC)	2.22	**Extinction[1,2]**	**481**	**±18**	**426**	**535**
		Non-extinction	409	±17	360	459
		Pseudorandom	408	±16	361	454
Dorsal frontal cortex (DFC)	2.22	**Extinction[1,2]**	**478**	**±21**	**416**	**540**
		Non-extinction	414	±19	359	470
		Pseudorandom	411	±18	358	464
Lateral frontal cortex (LFC)	2.22	**Extinction[2]**	**443**	**±21**	**381**	**506**
		Non-extinction	380	±19	324	436
		Pseudorandom	383	±18	330	436
Insular cortex, agranular (AI)	2.22	Extinction	398	±22	332	465
		Non-extinction	362	±20	302	422
		Pseudorandom	346	±19	289	402
Lateral orbital cortex (LO)	2.22	Extinction	659	±44	529	789
		Non-extinction	564	±39	447	681
		Pseudorandom	541	±37	431	652
Ventral orbital cortex (VO)	2.22	Extinction	635	±38	522	749
		Non-extinction	536	±34	434	638
		Pseudorandom	533	±33	437	630
Medial orbital cortex (MO)	2.22	Extinction	389	±24	319	460
		Non-extinction	342	±21	278	405
		Pseudorandom	338	±20	278	398
Infralimbic cortex (IL)	1.94	**Extinction[1]**	**364**	**±19**	**307**	**420**
		Non-extinction	323	±17	272	374
		Pseudorandom	295	±16	247	344
Granular insular cortex (GI)	1.10	Extinction	420	±23	350	490
		Non-extinction	375	±21	312	437
		Pseudorandom	357	±20	298	417
Anterior cingulate (Cg2)	1.10	Extinction	452	±23	383	521
		Non-extinction	410	±21	348	472
		Pseudorandom	389	±20	330	448
Accumbens, shell (AcbSh)	1.10	Extinction	311	±24	240	383
		Non-extinction	291	±22	227	355
		Pseudorandom	244	±20	183	304
Accumbens, core (AcbC)	1.10	Extinction	377	±24	305	449
		Non-extinction	343	±22	278	408
		Pseudorandom	308	±21	247	369

Caudate-putamen, rostral (rCPU)	1.10	**Extinction[1]**	**520**	**±28**	**437**	**603**
		Non-extinction	463	±25	389	538
		Pseudorandom	435	±24	365	506
Ventral diagonal band nucleus (VDB)	1.10	Extinction	331	±24	260	402
		Non-extinction	345	±21	281	409
		Pseudorandom	338	±20	278	399
Medial septal nucleus (MS)	1.10	Extinction	314	±17	263	365
		Non-extinction	332	±15	286	378
		Pseudorandom	327	±15	284	371
Lateral septal nucleus (LS)	1.10	Extinction	242	±18	190	295
		Non-extinction	278	±16	231	326
		Pseudorandom	264	±15	219	308
Parietal cortex, anterior (S1)	0.02	Extinction	482	±26	406	558
		Non-extinction	424	±23	356	493
		Pseudorandom	427	±22	362	492
Medial preoptic area (MPO)	0.02	Extinction	316	±24	244	388
		Non-extinction	326	±22	261	391
		Pseudorandom	279	±21	218	341
Lateral preoptic area (LPO)	0.02	Extinction	320	±23	253	387
		Non-extinction	302	±20	242	362
		Pseudorandom	287	±19	230	344
Horizontal limb of diagonal band, posterior (HDB)	0.02	Extinction	359	±22	293	424
		Non-extinction	331	±20	272	390
		Pseudorandom	327	±19	271	383
Caudate-putamen, middle (CPU)	0.02	Extinction	466	±23	398	533
		Non-extinction	425	±20	364	486
		Pseudorandom	409	±19	351	467
Posterior cingulate (CgP)	-1.22	Extinction	472	±21	410	535
		Non-extinction	488	±19	431	544
		Pseudorandom	477	±18	424	531
Parietal, medial (M1-2)	-1.22	**Extinction[1]**	**461**	**±11**	**430**	**492**
		Non-extinction	445	±11	414	476
		Pseudorandom	426	±12	392	459
Parietal, lateral (S1BF)	-1.22	Extinction	404	±30	316	492
		Non-extinction	429	±27	350	509
		Pseudorandom	396	±25	321	471
Perirhinal cortex, anterior (PRh)	-1.22	Extinction	315	±9	292	339
		Non-extinction[3]	**333**	**±9**	**309**	**357**
		Pseudorandom	292	±9	267	318
Centromedial nucleus, thalamus (CM)	-1.22	**Extinction[1]**	**457**	**±12**	**424**	**490**
		Non-extinction	436	±12	403	469
		Pseudorandom	415	±13	379	450
Medial dorsal nucleus, thalamus (MD)	-1.22	**Extinction[1]**	**459**	**±14**	**420**	**498**
		Non-extinction	443	±14	404	481
		Pseudorandom	409	±15	368	450
Medial dorsal lateral nucleus, thalamus (MDL)	-1.22	**Extinction[1]**	**508**	**±13**	**472**	**544**
		Non-extinction	481	±13	446	517
		Pseudorandom	450	±14	412	488

Central lateral nucleus, thalamus (CL)	-1.22	**Extinction[1]**	**519**	**±13**	**484**	**553**
		Non-extinction	493	±13	458	528
		Pseudorandom	472	±13	435	509
Ventral posterior lateral nucleus, thalamus (VPL)	-1.22	Extinction	435	±24	364	505
		Non-extinction	468	±21	405	531
		Pseudorandom	431	±20	371	491
Ventromedial nucleus, thalamus (VM)	-1.22	Extinction	502	±16	458	546
		Non-extinction	476	±16	432	520
		Pseudorandom	463	±17	416	510
Basolateral amygdala (BLA)	-1.22	Extinction	296	±18	243	348
		Non-extinction	320	±16	272	367
		Pseudorandom	304	±15	259	349
Central amygdala (CeA)	-1.22	Extinction	175	±15	130	219
		Non-extinction[4]	**226**	**±13**	**186**	**266**
		Pseudorandom	202	±13	164	240
Medial amygdala (MeA)	-1.22	Extinction	187	±15	143	231
		Non-extinction	229	±13	189	269
		Pseudorandom	210	±13	172	248
Ventromedial hypothalamus (VMH)	-1.22	Extinction	211	±15	166	257
		Non-extinction	238	±14	197	279
		Pseudorandom	222	±13	183	260
Caudate-putamen, caudal (cCPU)	-1.22	Extinction	285	±24	214	357
		Non-extinction	337	±22	272	401
		Pseudorandom	310	±20	250	371
Anterior hippocampus, CA1 (rCA1)	-1.94	Extinction	280	±16	232	328
		Non-extinction	287	±14	244	330
		Pseudorandom	282	±14	241	323
Anterior hippocampus, CA3 (rCA3)	-1.94	Extinction	352	±17	301	403
		Non-extinction	330	±15	284	376
		Pseudorandom	320	±15	276	363
Dentate gyrus (DG)	-1.94	Extinction	311	±14	269	353
		Non-extinction	317	±13	279	355
		Pseudorandom	306	±12	270	342
Hippocampal molecular layers (Mol)	-1.94	Extinction	344	±14	303	385
		Non-extinction	319	±12	283	356
		Pseudorandom	320	±12	286	355
Anterior pretectal area, dorsal (APTD)	-2.70	Extinction	429	±17	377	481
		Non-extinction	449	±16	402	496
		Pseudorandom	429	±15	385	473
Anterior pretectal area, ventral (APTV)	-2.70	Extinction	429	±15	384	475
		Non-extinction	458	±14	416	499
		Pseudorandom	425	±13	385	464
Lateral geniculate nucleus, thalamus (LGN)	-2.70	Extinction	409	±16	361	458
		Non-extinction	419	±15	376	463
		Pseudorandom	396	±14	355	437
Visual cortex (V1)	-2.70	Extinction	458	±16	409	507
		Non-extinction	441	±15	397	485
		Pseudorandom	445	±14	404	487

Ectorhinal cortex, posterior (Ect)	-2.70	Extinction	310	±22	244	375
		Non-extinction	351	±20	292	410
		Pseudorandom	350	±19	294	406
Deep entorhinal cortex (DEnt)	-2.70	Extinction	226	±12	190	263
		Non-extinction[4]	**279**	**±11**	**246**	**311**
		Pseudorandom	246	±10	215	277
Lateral entorhinal cortex (LEnt)	-2.70	Extinction	262	±18	208	317
		Non-extinction	293	±16	244	342
		Pseudorandom	295	±16	249	341
Ventral tegmental area (VTA)	-3.08	Extinction	269	±12	233	304
		Non-extinction[3,4]	**327**	**±11**	**295**	**359**
		Pseudorandom	287	±10	256	317
Mammillary bodies (MM)	-3.08	Extinction	562	±23	492	631
		Non-extinction	527	±21	465	590
		Pseudorandom	535	±20	476	595
Retrosplenial cortex (RSpl)	-3.08	Extinction	384	±16	351	417
		Non-extinction[3]	**406**	**±17**	**370**	**441**
		Pseudorandom	355	±18	319	391
Presubiculum (Psub)	-3.08	**Extinction[1]**	**400**	**±13**	**372**	**427**
		Non-extinction[3]	**414**	**±14**	**385**	**444**
		Pseudorandom	365	±15	334	395
Subiculum (Sub)	-3.08	**Extinction[1]**	**409**	**±14**	**381**	**437**
		Non-extinction[3]	**414**	**±15**	**383**	**444**
		Pseudorandom	370	±15	339	401
Posterior hippocampus, CA1 (cCA1)	-3.08	Extinction	330	±17	296	364
		Non-extinction	339	±18	302	376
		Pseudorandom	312	±19	274	350
Posterior hippocampus, CA2 (cCA2)	-3.08	Extinction	322	±12	297	347
		Non-extinction[3]	**342**	**±13**	**315**	**369**
		Pseudorandom	299	±13	272	327
Posterior hippocampus, CA3 (cCA3)	-3.08	**Extinction[1]**	**292**	**±11**	**270**	**313**
		Non-extinction[3]	**309**	**±12**	**286**	**333**
		Pseudorandom	263	±12	239	287
External cuneate nucleus (ECu)	-7.32	Extinction	578	±22	517	639
		Non-extinction[3]	**605**	**±22**	**545**	**665**
		Pseudorandom	538	±22	476	599
Cuneate nucleus (Cu)	-7.32	Extinction	546	±22	484	608
		Non-extinction	589	±22	528	650
		Pseudorandom	539	±23	477	602
Solitary tract nucleus (Sol)	-7.32	Extinction	509	±22	448	570
		Non-extinction	545	±22	485	605
		Pseudorandom	512	±22	451	574
Hypoglossal nucleus (12N)	-7.32	Extinction	456	±19	405	508
		Non-extinction	485	±18	434	536
		Pseudorandom	436	±19	384	488
Spinal trigeminal nucleus (SP5I)	-7.32	Extinction	327	±17	279	375
		Non-extinction	360	±17	312	407
		Pseudorandom	327	±18	278	376

Medullary reticular formation (Ret)	-7.32	Extinction	308	±16	264	351
		Non-extinction	341	±16	298	384
		Pseudorandom	311	±16	266	355
Cerebellum, vermis (CBV)	-7.32	Extinction	379	±16	335	423
		Non-extinction	402	±16	358	445
		Pseudorandom	365	±16	320	409
Cerebellum, lateral hemisphere (CBLH)	-7.32	Extinction	300	±15	258	342
		Non-extinction	326	±15	284	367
		Pseudorandom	292	±15	249	334
Caudate-putamen, rostral (rCPU), medial	1.10	**Extinction[1]**	**525**	**±28**	**443**	**608**
		Non-extinction	464	±25	390	538
		Pseudorandom	435	±24	365	506
Caudate-putamen, rostral (rCPU), lateral	1.10	Extinction	515	±28	430	599
		Non-extinction	463	±26	387	539
		Pseudorandom	435	±24	363	507

Bold[1], Extinction group greater than pseudorandom ($p<0.01$); **Bold[2]**, Extinction group greater than non-extinction group ($p<0.01$); **Bold[3]**, Non-extinction group greater than pseudorandom ($p<0.01$); **Bold[4]**, Non-extinction group greater than extinction group ($p<0.01$). Regions are listed by anterior-posterior Bregma coordinates, including the FDG uptake means (nCi/g tissue wet weight), standard errors, and 99% confidence intervals for each region by group.

Appendix Table 6.2: Results of covariance analysis (ANCOVA) of mean FDG uptake in auditory regions.

Region	Bregma	Group	Mean	S.E.	Lower 99%	Upper 99%
Auditory cortex, dorsal (TE1)	-3.08	Extinction	419	±19	380	458
		Non-extinction[3]	**429**	**±20**	**388**	**470**
		Pseudorandom	385	±19	346	423
Auditory cortex, ventral (TE3)	-3.08	Extinction	366	±19	327	406
		Non-extinction[3,4]	**411**	**±20**	**370**	**452**
		Pseudorandom	355	±19	315	394
Medial geniculate nucleus, dorsal (MGD)	-3.08	Extinction	359	±14	330	388
		Non-extinction[3]	**383**	**±14**	**355**	**412**
		Pseudorandom	333	±16	300	365
Medial geniculate nucleus, medial (MGM)	-3.08	Extinction	389	±13	362	416
		Non-extinction[3]	**410**	**±13**	**383**	**437**
		Pseudorandom	362	±15	332	393
Medial geniculate nucleus, ventral (MGV)	-3.08	**Extinction[1]**	**423**	**±14**	**394**	**451**
		Non-extinction[3]	**427**	**±14**	**399**	**456**
		Pseudorandom	383	±16	350	415
Lateral lemniscus nucleus, dorsal (DLL)	-4.60	Extinction	357	±20	315	399
		Non-extinction[3]	**373**	**±27**	**317**	**429**
		Pseudorandom	314	±25	263	365
Lateral lemniscus nucleus, intermediate (ILL)	-4.60	Extinction	411	±24	361	461
		Non-extinction[3,4]	**489**	**±32**	**421**	**556**
		Pseudorandom	377	±29	316	438
Lateral lemniscus nucleus, ventral (VLL)	-4.60	Extinction	415	±21	372	458
		Non-extinction[3,4]	**476**	**±28**	**418**	**535**
		Pseudorandom	380	±25	327	433
Inferior colliculus nucleus, dorsal (ICD)	-5.02	Extinction	382	±19	342	422
		Non-extinction	404	±20	363	444
		Pseudorandom	372	±21	328	416
Inferior colliculus nucleus, central (ICC)	-5.02	Extinction	749	±30	688	810
		Non-extinction[3]	**765**	**±30**	**702**	**828**
		Pseudorandom	689	±33	621	757
Inferior colliculus nucleus, external (ICE)	-5.02	Extinction	473	±23	426	519
		Non-extinction[3]	**509**	**±23**	**461**	**557**
		Pseudorandom	428	±25	376	480
Lateral superior olivary nucleus (LSO)	-5.34	Extinction	509	±25	457	560
		Non-extinction	528	±24	477	579
		Pseudorandom	499	±34	429	570
Medial superior olivary nucleus (MSO)	-5.34	Extinction	489	±22	443	535
		Non-extinction	514	±22	469	560
		Pseudorandom	457	±30	394	519
Trapezoid body nucleus (TBN)	-5.34	Extinction	339	±15	307	371
		Non-extinction[4]	**373**	**±15**	**342**	**405**
		Pseudorandom	342	±21	299	386

Ventral cochlear nucleus, anterior (VCA)	-5.34	Extinction	489	±17	453	526
		Non-extinction[3]	**515**	**±17**	**479**	**551**
		Pseudorandom	449	±22	401	497
Ventral cochlear nucleus, posterior (VCP)	-6.12	Extinction	498	±22	451	546
		Non-extinction[3]	**520**	**±22**	**473**	**567**
		Pseudorandom	436	±29	374	499
Dorsal cochlear nucleus (DCN)	-6.12	**Extinction[1]**	**563**	**±19**	**522**	**605**
		Non-extinction	561	±19	519	602
		Pseudorandom	508	±26	453	563

Bold[1], Extinction group greater than pseudorandom ($p<0.01$); **Bold[2]**, Extinction group greater than non-extinction group ($p<0.01$); **Bold[3]**, Non-extinction group greater than pseudorandom ($p<0.01$); **Bold[4]**, Non-extinction group greater than extinction group ($p<0.01$). Regions are listed by anterior-posterior Bregma coordinates, including the FDG uptake means (nCi/g tissue wet weight), standard errors, and 99% confidence intervals for each region by group.

Appendix Table 6.3: Reliably significant correlations by group.

Regions	Extinction	Non-extinction	Pseudorandom
DFC - VTA	**-0.99***	-0.88	-0.04
DFC - MD	**-0.99***	-0.27	-0.14
DFC - DCN	**-0.99***	0.52	0.60
DFC - MGD	**-0.99***	-0.90	0.73
DFC - TE1	**-0.99***	-0.68	0.82
MD - MDL	**0.92***	0.75	**0.90***
MD - CL	**0.91***	0.57	0.71
MD - MGV	**0.87***	0.09	0.21
MD - Sub	**0.89***	-0.03	0.30
MD - cCA2	**0.87***	-0.03	0.17
MDL - CL	**0.99***	**0.95***	**0.92***
MDL - CM	**0.91***	0.77	0.28
MDL - MGV	**0.93***	-0.06	0.24
MDL - Sub	**0.87***	0.22	0.47
MDL - cCA2	**0.89***	0.20	0.18
CL - CM	**0.90***	0.85	0.54
CL - MGV	**0.93***	-0.02	0.46
CL - Sub	**0.90***	0.29	0.60
CL - cCA2	**0.88***	0.21	0.31
CM - cCA2	**0.91***	-0.11	0.73
CM - cCA3	**0.88***	-0.12	0.36
cCA2 - cCA3	**0.88***	0.81	0.15
Sub - MGM	**0.95***	0.78	0.74
Sub - MGD	**0.88***	0.68	0.50
Sub - MGV	**0.91***	0.69	0.68
MGM - MGV	**0.90***	**0.98***	**0.94***
M1/M2 - Psub	**0.87***	-0.03	0.72
VCP - ICE	**0.95***	0.01	0.66
VCP - ICC	**0.96***	-0.28	-0.33
VCP - ILL	**0.92***	0.57	0.66
VCP - DLL	**0.93***	0.46	0.47
ICC - DLL	**0.91***	0.89	0.37
ICC - VLL	**0.93***	0.92	0.72
DLL - ILL	**0.91***	0.98	0.75
DLL - VLL	**0.94***	0.99	0.90
ILL - VLL	**0.89***	**0.98***	0.96
ECu - DLL	**0.93***	0.18	0.41
ECu - ILL	**0.87***	0.31	0.36
ECu - VLL	**0.92***	0.33	0.45
IL - rCPU	0.78	**0.90***	0.33
RSpl - rCPU	0.17	**-0.99***	0.35
MGD - MGV	0.76	**0.98***	0.82
MGD - MGM	0.83	**0.98***	0.75
CA2 - TE1	0.64	**0.96***	0.48
MFC - DFC	0.72	0.93	**0.96***
MFC - LFC	0.75	0.91	**0.94***
MFC - rCPU	0.42	0.81	**0.90***
DFC - LFC	0.96	0.95	**0.96***
rCPU - DFC	0.46	0.78	**0.94***
rCPU - LFC	0.66	0.85	**0.90***
rCPU - M1/M2	0.06	0.51	**0.94***
TE3 - ICC	-0.09	0.82	**0.96***

Pairwise inter-regional within-group correlations of FDG activity significant after jackknife at $p < .01$ are indicated in **bold***

Bibliography

Amaral, D. G. & Witter, M. P. (1995). Hippocampal formation. In R.P.Paxinos (Ed.), *The rat nervous system* (pp. 443-493). San Diego: Academic Press.

American Psychiatric Association (2000). *Diagnostic and statistical manual of mental disorders*. (4th ed. text revised ed.) Washington, DC: American Psychiatric Press.

Archer, J. (1975). Rodent sex differences in emotional and related behavior. *Behav.Biol., 14,* 451-479.

Bannerman, D. M., Deacon, R. M., Offen, S., Friswell, J., Grubb, M., & Rawlins, J. N. (2002). Double dissociation of function within the hippocampus: spatial memory and hyponeophagia. *Behav.Neurosci., 116,* 884-901.

Bannerman, D. M., Grubb, M., Deacon, R. M., Yee, B. K., Feldon, J., & Rawlins, J. N. (2003). Ventral hippocampal lesions affect anxiety but not spatial learning. *Behav.Brain Res., 139,* 197-213.

Barrett, D. & Gonzalez-Lima, F. (2004). Behavioral effects of metyrapone on Pavlovian extinction. *Neurosci.Lett., 371,* 91-96.

Barrett, D., Shumake, J., Jones, D., & Gonzalez-Lima, F. (2003). Metabolic mapping of mouse brain activity after extinction of a conditioned emotional response. *J.Neurosci., 23,* 5740-5749.

Berretta, S., Perciavalle, V., & Poppele, R. E. (1991). Origin of cuneate projections to the anterior and posterior lobes of the rat cerebellum. *Brain Res., 556,* 297-302.

Birke, L. I. & Archer, J. (1975). Open-field behaviour of oestrous and dioestrous rats: evidence against an 'emotionality' interpretation. *Anim Behav., 23,* 509-512.

Bohus, B. & De Kloet, E. R. (1981). Adrenal steroids and extinction behavior: antagonism by progesterone, deoxycorticosterone and dexamethasone of a specific effect of corticosterone. *Life Sci., 28,* 433-440.

Bouton, M. E. (1991). Context and retrieval in extinction and in other examples of interference in simple associative learning. In L.Dachowski & C. F. Flaherty (Eds.), *Current Topics in Animal Learning* (pp. 25-53). Hillsdale, New Jersey: Erlbaum.

Bouton, M. E. (2002). Context, ambiguity, and unlearning: sources of relapse after behavioral extinction. *Biol.Psychiatry, 52,* 976-986.

Brady, J. V. & Nauta, W. J. (1955). Subcortical mechanisms in emotional behavior: the duration of affective changes following septal and habenular lesions in the albino rat. *J.Comp.Physiol.Psychol., 48,* 412-420.

Bremner, J. D., Staib, L. H., Kaloupek, D., Southwick, S. M., Soufer, R., & Charney, D. S. (1999). Neural correlates of exposure to traumatic pictures and sound in Vietnam combat veterans with and without posttraumatic stress disorder: a positron emission tomography study. *Biol.Psychiatry, 45,* 806-816.

Cahill, L. & McGaugh, J. L. (1998). Mechanisms of emotional arousal and lasting declarative memory. *Trends Neurosci., 21,* 294-299.

Caldecott-Hazard, S., Mazziotta, J., & Phelps, M. (1988). Cerebral correlates of depressed behavior in rats, visualized using 14C-2-deoxyglucose autoradiography. *J.Neurosci., 8,* 1951-1961.

Carlson, N. R. (2004). *Physiology of behavior*. (8th ed.) Boston: Pearson Education, Inc.

Charney, D. S., Deutch, A. Y., Krystal, J. H., Southwick, S. M., & Davis, M. (1993). Psychobiologic mechanisms of posttraumatic stress disorder. *Arch.Gen.Psychiatry, 50,* 294-305.

Christofi, G., Nowicky, A. V., Bolsover, S. R., & Bindman, L. J. (1993). The postsynaptic induction of nonassociative long-term depression of excitatory synaptic transmission in rat hippocampal slices. *J.Neurophysiol., 69,* 219-229.

Coover, G. D., Goldman, L., & Levine, S. (1971). Plasma corticosterone levels during extinction of a lever-press response in hippocampectomized rats. *Physiol Behav., 7,* 727-732.

Corcoran, K. A. & Maren, S. (2001). Hippocampal inactivation disrupts contextual retrieval of fear memory after extinction. *J.Neurosci., 21,* 1720-1726.

Corcoran, K. A. & Maren, S. (2004). Factors regulating the effects of hippocampal inactivation on renewal of conditional fear after extinction. *Learn.Mem., 11,* 598-603.

Cordero, M. I., Kruyt, N. D., Merino, J. J., & Sandi, C. (2002). Glucocorticoid involvement in memory formation in a rat model for traumatic memory. *Stress., 5,* 73-79.

Davis, M., Falls, W. A., Campeau, S., & Kim, M. (1993). Fear-potentiated startle: a neural and pharmacological analysis. *Behav.Brain Res., 58,* 175-198.

Dean, C. & Kostreva, D. R. (1987). Cardiac, respiratory, and renal responses to stimulation of the external cuneate nucleus. *J.Appl.Physiol, 63,* 158-166.

Delahanty, D. L., Raimonde, A. J., & Spoonster, E. (2000). Initial posttraumatic urinary cortisol levels predict subsequent PTSD symptoms in motor vehicle accident victims. *Biol.Psychiatry, 48,* 940-947.

Domjan, M. (2002). *The principles of learning and behavior*. (5th ed.) Pacific Grove, CA: Brooks/Cole.

Domjan, M., Cusato, B., & Villarreal, R. (2000). Pavlovian feed-forward mechanisms in the control of social behavior. *Behav.Brain Sci., 23,* 235-249.

Edwards, E., Harkins, K., Wright, G., & Henn, F. (1990). Effects of bilateral adrenalectomy on the induction of learned helplessness behavior. *Neuropsychopharmacology, 3,* 109-114.

Edwards, E., King, J. A., & Fray, J. (2000). Hypertension and insulin resistant models have divergent propensities to learned helpless behavior in rodents. *Am.J.Hypertens., 13,* 659-665.

Estes, W. K. & Skinner, B. F. (1941). Some quantitative properties of anxiety. *J.Exp.Psychol., 29,* 390-400.

Falls, W. A. (1998). Extinction: a review of theory and the evidence suggesting that memories are not erased with nonreinforcement. In W.O'Donohue (Ed.), *Learning and behavior therapy* (pp. 205-229). Boston: Allyn and Bacon.

Fanselow, M. S. (1989). The adaptive function of conditioned defensive behavior: an ecological approach to Pavlovian stimulus-substitution theory. In R.J.Blanchard, P. F. Brain, D. C. Blanchard, & S. Parmigiani (Eds.), *Ethoexperimental approaches to the study of behavior* (pp. 151-166). Boston: Kluwer.

Freedman, L. J., Insel, T. R., & Smith, Y. (2000). Subcortical projections of area 25 (subgenual cortex) of the macaque monkey. *J.Comp Neurol., 421,* 172-188.

Furchtgott, E., Wechkin, S., & Dees, J. W. (1961). Open-field exploration as a function of age. *J.Comp.Physiol.Psychol., 54,* 386-388.

Ganguli, R., Mintun, M. A., Becker, J. T., Brar, J. S., Diehl, D., Deleo, M. et al. (1994). Effects of hydrocortisone infusion on rCBF in schizophrenic patients during a memory task. An O15 PET Study. *Ann.N.Y.Acad.Sci., 746,* 385-387.

Garrud, P., Gray, J. A., & de Wied, D. (1974). Pituitary-adrenal hormones and extinction of rewarded behaviour in the rat. *Physiol Behav., 12,* 109-119.

Gewirtz, J. C., Falls, W. A., & Davis, M. (1997). Normal conditioned inhibition and extinction of freezing and fear-potentiated startle following electrolytic lesions of medical prefrontal cortex in rats. *Behav.Neurosci., 111,* 712-726.

Gilboa, A., Shalev, A. Y., Laor, L., Lester, H., Louzoun, Y., Chisin, R. et al. (2004). Functional connectivity of the prefrontal cortex and the amygdala in posttraumatic stress disorder. *Biol.Psychiatry, 55,* 263-272.

Goddard, G. V. (1964). Functions of the amygdala. *Psychol.Bull., 62,* 89-109.

Gonzalez-Lima, F. (1992). Brain imaging of auditory learning functions in rats: studies with fluorodeoxyglucose autoradiography and cytochrome oxidase histochemistry. In F.Gonzalez-Lima, T. Finkenstadt, & H. Scheich (Eds.), *Advances in metabolic mapping techniques for brain imaging of behavioral and learning functions* (pp. 39-109). Boston: Kluwer.

Gonzalez-Lima, F. & Bruchey, A. K. (2004). Extinction memory improvement by the metabolic enhancer methylene blue. *Learn.Mem., 11,* 633-640.

Gonzalez-Lima, F., Finkenstadt, T., & Ewert, J. P. (1989a). Learning-related activation in the auditory system of the rat produced by long-term habituation: a 2-deoxyglucose study. *Brain Res., 489,* 67-79.

Gonzalez-Lima, F., Finkenstadt, T., & Ewert, J. P. (1989b). Neural substrates for long-term habituation of the acoustic startle reflex in rats: a 2-deoxyglucose study. *Neurosci.Lett., 96,* 151-156.

Haynes Jr., R. C. (1990). Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In A.Goodman & L. Gillman (Eds.), *The pharmacological basis of therapeutics* (pp. 1431-1462). New York: Pergamon Press.

Heimer, L., Zahm, D. S., & Alheid, G. F. (1995). Basal ganglia. In R.P.Paxinos (Ed.), *The rat nervous system* (pp. 579-628). San Diego: Academic Press.

Helmstetter, F. J. (1992). Contribution of the amygdala to learning and performance of conditional fear. *Physiol Behav., 51,* 1271-1276.

Henke, P. G. (1990). Hippocampal pathway to the amygdala and stress ulcer development. *Brain Res.Bull., 25,* 691-695.

Henn, F. A. & Edwards, E. (1994). Animal models in the study of genetic factors in human psychopathology. In D.F.Papolos & H. M. Lachman (Eds.), *Genetic studies in affective disorders: overview of basic methods, current directions, and critical research issues* (New York: John Wiley and Sons.

Henn, F. A., Edwards, E., & Muneyyirci, J. (1993). Animal models of depression. *Clin.Neurosci., 1,* 152-156.

Henn, F. A., Johnson, J., Edwards, E., & Anderson, D. (1985). Melancholia in rodents: neurobiology and pharmacology. *Psychopharmacol.Bull., 21,* 443-446.

Hennessy, J. W., Cohen, M. E., & Rosen, A. J. (1973). Adrenocortical influences upon the extinction of an appetitive runway response. *Physiol Behav., 11,* 767-770.

Hernandez-Poudevida, P., McEwen, B. S., & Quirk, G. J. (2002). Inhibition of corticosterone with metyrapone prevents extinction of the conditioned emotional response (CER). *Soc.Neurosci.(Abstr.), 83.15*.

Herry, C. & Garcia, R. (2002). Prefrontal cortex long-term potentiation, but not long-term depression, is associated with the maintenance of extinction of learned fear in mice. *J.Neurosci., 22,* 577-583.

Herry, C., Vouimba, R. M., & Garcia, R. (1999). Plasticity in the mediodorsal thalamo-prefrontal cortical transmission in behaving mice. *J.Neurophysiol., 82,* 2827-2832.

Hui, G. K., Figueroa, I. R., Poytress, B. S., Roozendaal, B., McGaugh, J. L., & Weinberger, N. M. (2004). Memory enhancement of classical fear conditioning by post-training injections of corticosterone in rats. *Neurobiol.Learn.Mem., 81,* 67-74.

Jacobson, L. & Sapolsky, R. (1991). The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr.Rev., 12,* 118-134.

Jones, D. & Gonzalez-Lima, F. (2001a). Associative effects of Pavlovian differential inhibition of behaviour. *Eur.J.Neurosci., 14,* 1915-1927.

Jones, D. & Gonzalez-Lima, F. (2001b). Mapping Pavlovian conditioning effects on the brain: blocking, contiguity, and excitatory effects. *J.Neurophysiol., 86,* 809-823.

Kamin, L. J. (1969). Predictability, surprise, attention, and conditioning. In B.A.Campbell & R. M. Church (Eds.), *Punishment and Aversive Behavior* (pp. 242-259). New York: Appleton-Century-Crofts.

Kim, J. J., Rison, R. A., & Fanselow, M. S. (1993). Effects of amygdala, hippocampus, and periaqueductal gray lesions on short- and long-term contextual fear. *Behav.Neurosci., 107,* 1093-1098.

King, J. A., Abend, S., & Edwards, E. (2001). Genetic predisposition and the development of posttraumatic stress disorder in an animal model. *Biol.Psychiatry, 50,* 231-237.

Kjelstrup, K. G., Tuvnes, F. A., Steffenach, H. A., Murison, R., Moser, E. I., & Moser, M. B. (2002). Reduced fear expression after lesions of the ventral hippocampus. *Proc.Natl.Acad.Sci.U.S.A, 99,* 10825-10830.

Krettek, J. E. & Price, J. L. (1977). Projections from the amygdaloid complex and adjacent olfactory structures to the entorhinal cortex and to the subiculum in the rat and cat. *J.Comp Neurol., 172,* 723-752.

Lan, C. T., Wen, C. Y., Tseng, G. F., Tan, C. K., Ling, E. A., & Shieh, J. Y. (1994). Efferent connections from the external cuneate nucleus to the medulla oblongata in the gerbil. *Brain Res., 668,* 107-116.

LeDoux, J. E. (1995). Emotion: clues from the brain. *Annu.Rev.Psychol., 46,* 209-235.

Liu, L., Tsuji, M., Takeda, H., Takada, K., & Matsumiya, T. (1999). Adrenocortical suppression blocks the enhancement of memory storage produced by exposure to psychological stress in rats. *Brain Res., 821,* 134-140.

Loscertales, M., Rose, S. P., & Sandi, C. (1997). The corticosteroid synthesis inhibitors metyrapone and aminoglutethimide impair long-term memory for a passive avoidance task in day-old chicks. *Brain Res., 769,* 357-361.

Lupien, S. J. & McEwen, B. S. (1997). The acute effects of corticosteroids on cognition: integration of animal and human model studies. *Brain Res.Brain Res.Rev., 24,* 1-27.

Lupien, S. J., Wilkinson, C. W., Briere, S., Menard, C., Ng Ying Kin, N. M., & Nair, N. P. (2002). The modulatory effects of corticosteroids on cognition: studies in young human populations. *Psychoneuroendocrinology, 27,* 401-416.

Mantle-St John, L. A. & Tracey, D. J. (1987). Somatosensory nuclei in the brainstem of the rat: independent projections to the thalamus and cerebellum. *J.Comp Neurol., 255,* 259-271.

Maren, S. (1999). Neurotoxic or electrolytic lesions of the ventral subiculum produce deficits in the acquisition and expression of Pavlovian fear conditioning in rats. *Behav.Neurosci., 113,* 283-290.

Maren, S., Aharonov, G., & Fanselow, M. S. (1997). Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats. *Behav.Brain Res., 88,* 261-274.

McCaughey, S. A., Giza, B. K., Nolan, L. J., & Scott, T. R. (1997). Extinction of a conditioned taste aversion in rats: II. Neural effects in the nucleus of the solitary tract. *Physiol Behav., 61,* 373-379.

Mcdonald, A. J., Mascagni, F., & Guo, L. (1996). Projections of the medial and lateral prefrontal cortices to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat. *Neuroscience, 71,* 55-75.

McEwen, B. S., De Kloet, E. R., & Rostene, W. (1986). Adrenal steroid receptors and actions in the nervous system. *Physiol Rev., 66,* 1121-1188.

McGeorge, A. J. & Faull, R. L. (1989). The organization of the projection from the cerebral cortex to the striatum in the rat. *Neuroscience, 29,* 503-537.

McHugh, S. B., Deacon, R. M., Rawlins, J. N., & Bannerman, D. M. (2004). Amygdala and ventral hippocampus contribute differentially to mechanisms of fear and anxiety. *Behav.Neurosci., 118,* 63-78.

McIntosh, A. R. & Gonzalez-Lima, F. (1993). Network analysis of functional auditory pathways mapped with fluorodeoxyglucose: associative effects of a tone conditioned as a Pavlovian excitor or inhibitor. *Brain Res., 627,* 129-140.

McIntosh, A. R. & Gonzalez-Lima, F. (1994). Network interactions among limbic cortices, basal forebrain, and cerebellum differentiate a tone conditioned as a Pavlovian excitor or inhibitor: fluorodeoxyglucose mapping and covariance structural modeling. *J.Neurophysiol., 72,* 1717-1733.

McIntosh, A. R., Rajah, M. N., & Lobaugh, N. J. (1999). Interactions of prefrontal cortex in relation to awareness in sensory learning. *Science, 284,* 1531-1533.

McNally, G. P., Pigg, M., & Weidemann, G. (2004). Opioid receptors in the midbrain periaqueductal gray regulate extinction of Pavlovian fear conditioning. *J.Neurosci., 24,* 6912-6919.

Milad, M. R. & Quirk, G. J. (2002). Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature, 420,* 70-74.

Miller, R. R. & Spear, N. E. (1985). *Information Processing in Animals: Conditioned Inhibition*. New Jersey: L. Erlbaum Associates.

Mizoguchi, K., Ishige, A., Takeda, S., Aburada, M., & Tabira, T. (2004). Endogenous glucocorticoids are essential for maintaining prefrontal cortical cognitive function. *J.Neurosci., 24,* 5492-5499.

Molchan, S. E., Sunderland, T., McIntosh, A. R., Herscovitch, P., & Schreurs, B. G. (1994). A functional anatomical study of associative learning in humans. *Proc.Natl.Acad.Sci.U.S.A, 91,* 8122-8126.

Morgan, M. A. & LeDoux, J. E. (1995). Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats. *Behav.Neurosci., 109,* 681-688.

Morgan, M. A., Romanski, L. M., & LeDoux, J. E. (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neurosci.Lett., 163,* 109-113.

Morgan, M. A., Schulkin, J., & LeDoux, J. E. (2003). Ventral medial prefrontal cortex and emotional perseveration: the memory for prior extinction training. *Behav.Brain Res., 146,* 121-130.

Morris, J. S., Smith, K. A., Cowen, P. J., Friston, K. J., & Dolan, R. J. (1999). Covariation of activity in habenula and dorsal raphe nuclei following tryptophan depletion. *Neuroimage, 10,* 163-172.

Nair, H. P., Berndt, J. D., Barrett, D., & Gonzalez-Lima, F. (2001a). Maturation of extinction behavior in infant rats: large-scale regional interactions with medial prefrontal cortex, orbitofrontal cortex, and anterior cingulate cortex. *J.Neurosci., 21,* 4400-4407.

Nair, H. P., Berndt, J. D., Barrett, D., & Gonzalez-Lima, F. (2001b). Metabolic mapping of brain regions associated with behavioral extinction in preweanling rats. *Brain Res., 903,* 141-153.

Nair, H. P. & Gonzalez-Lima, F. (1999). Extinction of behavior in infant rats: development of functional coupling between septal, hippocampal, and ventral tegmental regions. *J.Neurosci., 19,* 8646-8655.

Nobrega, J. N. (1992). Brain metabolic mapping and behavior: assessing the effects of early developmental experiences in adult animals. In F.Gonzalez-Lima, T. Finkenstadt, & H. Scheich (Eds.), *Advances in metabolic mapping techniques for brain imaging of behavioral and learning functions* (pp. 125-149). Boston: Kluwer.

Oitzl, M. S. & De Kloet, E. R. (1992). Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. *Behav.Neurosci., 106,* 62-71.

Orr, S. P., Metzger, L. J., Lasko, N. B., Macklin, M. L., Peri, T., & Pitman, R. K. (2000). De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. *J.Abnorm.Psychol., 109,* 290-298.

Overmier, J. B. & Seligman, M. E. (1967). Effects of inescapable shock upon subsequent escape and avoidance responding. *J.Comp Physiol Psychol., 63,* 28-33.

Pare, D., Quirk, G. J., & Ledoux, J. E. (2004). New vistas on amygdala networks in conditioned fear. *J.Neurophysiol., 92,* 1-9.

Pavlov, I. P. (1927). *Conditioned reflexes*. London: Oxford UP.

Paxinos, G. & Franklin, K. (2001). *The mouse brain in stereotaxic coordinates*. (2nd ed.) San Diego: Academic.

Peri, T., Ben Shakhar, G., Orr, S. P., & Shalev, A. Y. (2000). Psychophysiologic assessment of aversive conditioning in posttraumatic stress disorder. *Biol.Psychiatry, 47,* 512-519.

Petrovich, G. D., Canteras, N. S., & Swanson, L. W. (2001). Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. *Brain Res.Brain Res.Rev., 38,* 247-289.

Pitman, R. K., van der Kolk, B. A., Orr, S. P., & Greenberg, M. S. (1990). Naloxone-reversible analgesic response to combat-related stimuli in posttraumatic stress disorder. A pilot study. *Arch.Gen.Psychiatry, 47,* 541-544.

Poremba, A. & Gabriel, M. (1997). Amygdalar lesions block discriminative avoidance learning and cingulothalamic training-induced neuronal plasticity in rabbits. *J.Neurosci., 17,* 5237-5244.

Pugh, C. R., Fleshner, M., & Rudy, J. W. (1997). Type II glucocorticoid receptor antagonists impair contextual but not auditory-cue fear conditioning in juvenile rats. *Neurobiol.Learn.Mem., 67,* 75-79.

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *J.Neurosci., 20,* 6225-6231.

Rescorla, R. A. (1997). Spontaneous recovery after Pavlovian conditioning with multiple outcomes. *Anim.Learn.Behav., 25,* 99-107.

Rescorla, R. A. & Wagner, A. R. (1972). A theory of Pavlovian conditioning: variations in the effectiveness of reinforcement and nonreinforcement. In A.H.Black & W. F. Prokasy (Eds.), *Classical conditioning II* (pp. 64-99). New York: Appleton-Century-Crofts.

Richards, W. G., Farley, J., & Alkon, D. L. (1984). Extinction of associative learning in Hermissenda: behavior and neural correlates. *Behav.Brain Res., 14,* 161-170.

Richardson, R., Ledgerwood, L., & Cranney, J. (2004). Facilitation of fear extinction by D-cycloserine: theoretical and clinical implications. *Learn.Mem., 11,* 510-516.

Richmond, M. A., Yee, B. K., Pouzet, B., Veenman, L., Rawlins, J. N., Feldon, J. et al. (1999). Dissociating context and space within the hippocampus: effects of complete, dorsal, and ventral excitotoxic hippocampal lesions on conditioned freezing and spatial learning. *Behav.Neurosci., 113,* 1189-1203.

Roozendaal, B., Bohus, B., & McGaugh, J. L. (1996). Dose-dependent suppression of adrenocortical activity with metyrapone: effects on emotion and memory. *Psychoneuroendocrinology, 21,* 681-693.

Roozendaal, B., Carmi, O., & McGaugh, J. L. (1996). Adrenocortical suppression blocks the memory-enhancing effects of amphetamine and epinephrine. *Proc.Natl.Acad.Sci.U.S.A, 93,* 1429-1433.

Rotllant, D., Ons, S., Carrasco, J., & Armario, A. (2002). Evidence that metyrapone can act as a stressor: effect on pituitary-adrenal hormones, plasma glucose and brain c-fos induction. *Eur.J.Neurosci., 16,* 693-700.

Schreurs, B. G., Bahro, M., Molchan, S. E., Sunderland, T., & McIntosh, A. R. (2001). Interactions of prefrontal cortex during eyeblink conditioning as a function of age. *Neurobiol.Aging., 22,* 237-246.

Seliger, D. L. (1977). Effects of age, sex, and brightness of rield on open-field behaviors of rats. *Percept.Mot.Skills, 45,* 1059-1067.

Sesack, S. R., Deutch, A. Y., Roth, R. H., & Bunney, B. S. (1989). Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: an anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *J.Comp Neurol., 290,* 213-242.

Sharp, F. R., Sagar, S. M., & Swanson, R. A. (1993). Metabolic mapping with cellular resolution: c-fos vs. 2-deoxyglucose. *Crit Rev.Neurobiol., 7,* 205-228.

Shumake, J., Conejo-Jimenez, N., Gonzalez-Pardo, H., & Gonzalez-Lima, F. (2004). Brain differences in newborn rats predisposed to helpless and depressive behavior. *Brain Res., 1030,* 267-276.

Shumake, J., Edwards, E., & Gonzalez-Lima, F. (2001). Hypermetabolism of paraventricular hypothalamus in the congenitally helpless rat. *Neurosci.Lett., 311,* 45-48.

Shumake, J., Edwards, E., & Gonzalez-Lima, F. (2002). Dissociation of septo-hippocampal metabolism in the congenitally helpless rat. *Neuroscience, 114,* 373-377.

Shumake, J., Edwards, E., & Gonzalez-Lima, F. (2003a). Opposite metabolic changes in the habenula and ventral tegmental area of a genetic model of helpless behavior. *Brain Res., 963,* 274-281.

Shumake, J. & Gonzalez-Lima, F. (2003b). Brain systems underlying susceptibility to helplessness and depression. *Behav.Cogn Neurosci.Rev., 2,* 198-221.

Shumake, J., Poremba, A., Edwards, E., & Gonzalez-Lima, F. (2000). Congenital helpless rats as a genetic model for cortex metabolism in depression. *Neuroreport, 11,* 3793-3798.

Slotnick, B. M. & Leonard, C. M. (1975). *A stereotaxic atlas of the albino mouse forebrain*. Rockville, MD: Department of Health, Education and Welfare.

Smotherman, W. P. & Levine, S. (1978). ACTH effects on response suppression and plasma corticosterone in the mouse. *Physiol Behav., 20,* 503-507.

Sokoloff, L. (1992). Imaging techniques in studies of neural functions. In F.Gonzalez-Lima, T. Finkenstadt, & H. Scheich (Eds.), *Advances in metabolic mapping techniques for brain imaging of behavior and learning functions* (pp. 1-37). Boston: Kluwer.

Strashimirov, D. & Bohus, B. (1966). Effect of 2-methyl-1,2-bis-3-pyridl-1-propanone (SU-4885) on adrenocortical secretion in normal and hypophysectomized rats. *Steroids, 7,* 171-180.

Swanson, L. W. & Cowan, W. M. (1977). An autoradiographic study of the organization of the efferent connections of the hippocampal formation in the rat. *J.Comp Neurol., 172,* 49-84.

Szapiro, G., Vianna, M. R., McGaugh, J. L., Medina, J. H., & Izquierdo, I. (2003). The role of NMDA glutamate receptors, PKA, MAPK, and CAMKII in the hippocampus in extinction of conditioned fear. *Hippocampus, 13,* 53-58.

Treit, D. & Fundytus, M. (1988). Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacol.Biochem.Behav., 31,* 959-962.

van Groen, T. & Wyss, J. M. (1990). Extrinsic projections from area CA1 of the rat hippocampus: olfactory, cortical, subcortical, and bilateral hippocampal formation projections. *J.Comp Neurol., 302,* 515-528.

Vermetten, E. & Bremner, J. D. (2002). Circuits and systems in stress. II. Applications to neurobiology and treatment in posttraumatic stress disorder. *Depress.Anxiety., 16,* 14-38.

Vollmayr, B., Bachteler, D., Vengeliene, V., Gass, P., Spanagel, R., & Henn, F. (2004). Rats with congenital learned helplessness respond less to sucrose but show no deficits in activity or learning. *Behav.Brain Res., 150,* 217-221.

Vouimba, R. M., Garcia, R., Baudry, M., & Thompson, R. F. (2000). Potentiation of conditioned freezing following dorsomedial prefrontal cortex lesions does not interfere with fear reduction in mice. *Behav.Neurosci., 114,* 720-724.

White, N. M. & Salinas, J. A. (2003). Mnemonic functions of dorsal striatum and hippocampus in aversive conditioning. *Behav.Brain Res., 142,* 99-107.

Yehuda, R. (1999). Linking the neuroendocrinology of post-traumatic stress disorder with recent neuroanatomic findings. *Semin.Clin.Neuropsychiatry, 4,* 256-265.

Zorawski, M. & Killcross, S. (2002). Posttraining glucocorticoid receptor agonist enhances memory in appetitive and aversive Pavlovian discrete-cue conditioning paradigms. *Neurobiol.Learn.Mem., 78,* 458-464.

Vita

Douglas Wayne Barrett was born in Brazosport, TX, on March 22nd, 1975, the son of Carl Barrett and Carol Cook. He graduated as salutatorian from Cheshire High School in Cheshire, CT, in May of 1993. He then attended the Massachusetts Institute of Technology in Cambridge, MA, and graduated with a B.S. in Brain and Cognitive Sciences. In August of 1998, he entered the Graduate School of the University of Texas at Austin.

Permanent address: 501 W 26th St, Austin, TX, 78705

This dissertation was typed by the author.